

Investigating the role of scaffolds for rotator cuff repair

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Declaration

I certify that this thesis is true and honest account of my own research, which was conducted ethically and the results obtained are genuine. Where I have drawn on the work, ideas, and results of others, this has been appropriately acknowledged in the thesis.

Richard David James Smith

Abstract

Surgical repairs of large-massive rotator cuff tendon tears often fail. Scaffolds can be used to augment tendon repair, but few scaffolds have demonstrated clinical efficacy. The aim of this thesis was to evaluate and compare several different types of commercially available scaffolds. Three important aspects of scaffold performance were studied: mechanical properties, tenocyte response, and macrophage response.

In terms of mechanical properties, synthetic scaffolds had superior macroscale mechanical properties compared to biological scaffolds, but remained significantly weaker than native human supraspinatus tendon. The microscale mechanical properties of biological scaffolds were similar to those of the human supraspinatus tendon, but synthetic scaffolds were significantly stiffer on the microscale. None of the tested scaffolds mimicked the mechanical properties of human supraspinatus tendon on both the macroscale and microscale.

The tenocyte study revealed that more cells initially attached to synthetic scaffolds with aligned fibres. However, over 13 days, the human biological scaffold promoted the greatest tenocyte proliferation. The non-cross-linked porcine biological scaffold stimulated the highest expression of *COL1A1* and *COL3A1* mRNA.

Human macrophages generated a pro-inflammatory response towards the non-cross-linked porcine biological scaffold tested. The cross-linked porcine biological scaffold prompted a chronic inflammatory response, whereas the human biological and synthetic scaffolds did not induce an overt inflammatory response.

This study highlights significant variation in the properties and performance of different commercially available scaffolds. It also illustrates the inadequacy of many existing scaffolds to successfully augment a surgical rotator cuff repair. Future scaffolds should be specifically designed to meet the mechanical and biological demands of the target tissue. The pipeline of *in vitro* tests developed in this thesis could be used to screen new scaffolds and help determine which scaffolds to evaluate in animal models and human trials.

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Abbreviations

AAOS	American academy of orthopaedic surgeons
ACAN	Aggrecan
ACTB	Beta-actin
AFM	Atomic force microscopy
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenic protein
CI	Confidence Interval
COL1A1	Collagen type 1
COL2A1	Collagen type 2
COL3A1	Collagen type 3
COL6A1	Collagen type 6
CSF2	Colony stimulating factor 2
DAPI	4', 6-Diamidino-2-Phenylindole
DCN	Decorin
dsDNA	Double stranded deoxyribonucleic acid
ECM	Extracellular matrix
ESEM	Environmental scanning electron microscopy
FBGC	Foreign body giant cell
FBR	Foreign body reaction
FBS	Fetal bovine serum
GAG	Glycosamnioglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDMI	Hexamethylene diisocyanate
IFN- γ	Interferon gamma
IGF-1	Insulin-like growth factor
IL-10	Interleukin 10
IL-1 β	Interleukin-1 beta
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinase
MMP-1	Matrix metalloproteinase-1
MMP-3	Matrix metalloproteinase-3
MRI	Magnetic resonance imaging
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PET	Polyethylene terephthalate
PGA	Polyglycolic acid
PH4B	Poly-4-hydroxybutyrate
PLA	Polylactic acid
PLLA	Poly-L-lactic acid

PRP	Platelet rich plasma
SAD	Subacromial decompression
SCX-B	Scleraxis-B
SEM	Scanning electron microscopy
SIS	Small intestine submucosa
SPM	Scanning probe microscopy
TACE	TNF- α converting enzyme
TBP	TATA-box binding protein
TCP	Tissue culture plastic
TE	Tris-EDTA
TGF- β	Transforming growth factor-beta
TIMP	Tissue inhibitor of matrix metalloproteinase
TNC	Tenascin C
TNF- α	Tumor necrosis factor alpha
TNMD	Tenomodulin
VCAN	Versican
VEGF	Vascular endothelial growth factor
α -gal	Galactose-alpha-1,3-galactose

Chapter 1 : Introduction

1.1 The healthy shoulder

The shoulder is the most mobile part of the human body. It is composed of four joints including the acromioclavicular joint, the sternoclavicular joint, the scapulothoracic joint, and the glenohumeral joint (figure 1-1). The glenohumeral joint, often referred to as the 'shoulder joint' is the ball and socket joint that connects the head of the humerus to the glenoid fossa of the scapula (figure 1-1). The humeral head lies against, not within, the glenoid fossa, permitting a large range of movement at the cost of decreased stability. The shoulder is stabilized by ligaments (figure 1-2), tendons (figure 1-3), and the glenoid labrum (figure 1-1), a fibrocartilaginous structure that overlies the edge of the glenoid fossa and deepens the joint by 50% (1).

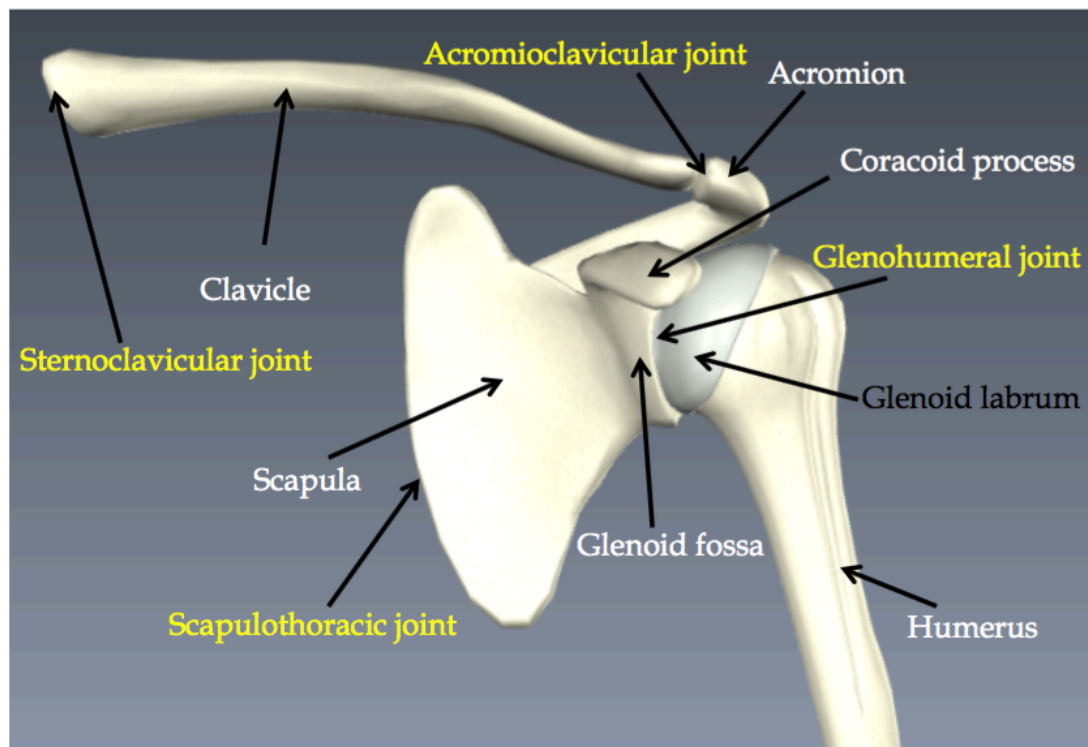


Figure 1-1. Bones, joints, and the labrum of the left shoulder (anterior view).

Image was taken from www.biodigital.com (2). Bones are labelled in white, joints are labelled in yellow, and the labrum is labelled in black.

Several ligaments help to stabilise the shoulder, while bursae help to reduce friction and cushion impacts (figure 1-2).

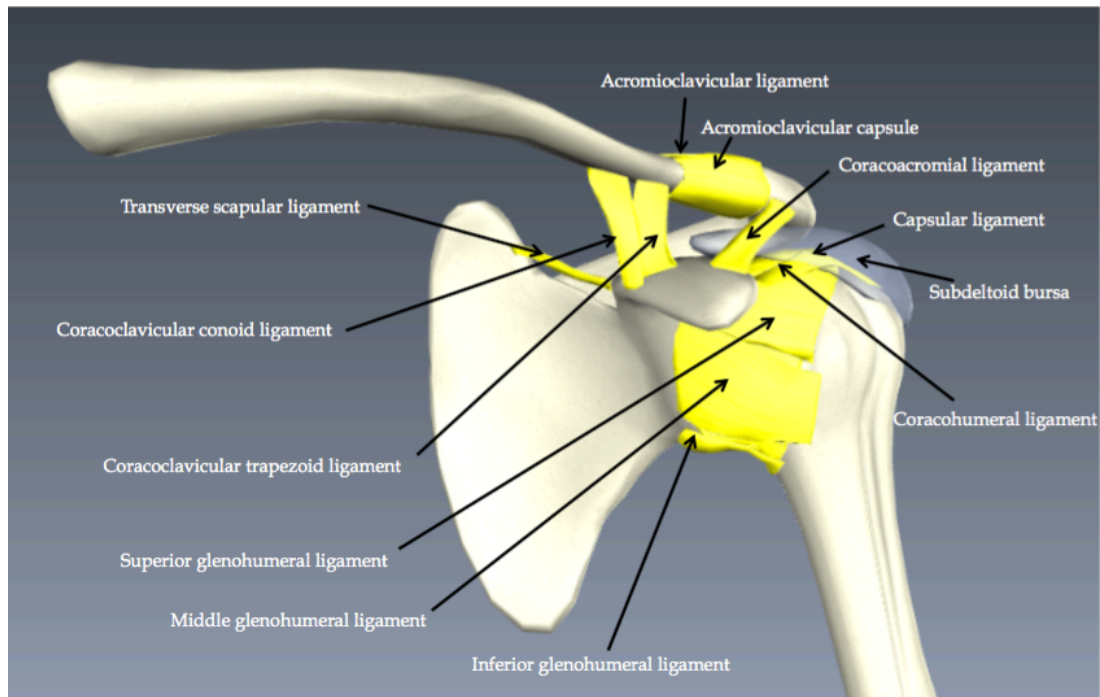


Figure 1-2. Ligaments and bursae of the left shoulder (anterior view).

Image was taken from www.biodigital.com (2). Ligaments and bursae are labelled in white.

1.1.1 Macroscopic structure of the rotator cuff

The supraspinatus, infraspinatus, teres minor, and subscapularis are the four muscles of the rotator cuff (figures 1-3 and 1-4). They all originate from the scapula and insert onto the head of the humerus. Close to the glenohumeral joint, all four tendons of the rotator cuff muscles inter-digitate to form a continuous 'cuff' around the humeral head.

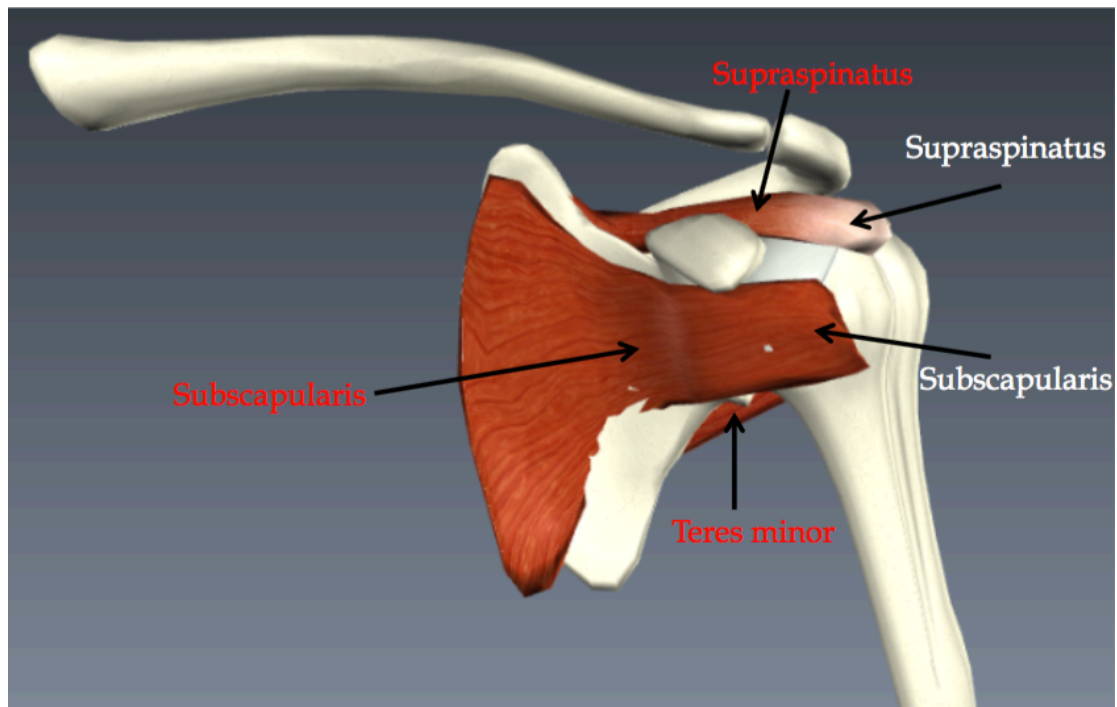


Figure 1-3. Rotator cuff muscles and tendons of the left shoulder (anterior view).

Image was taken from www.biodigital.com (2). Muscles are labelled in red and tendons are labelled in white.

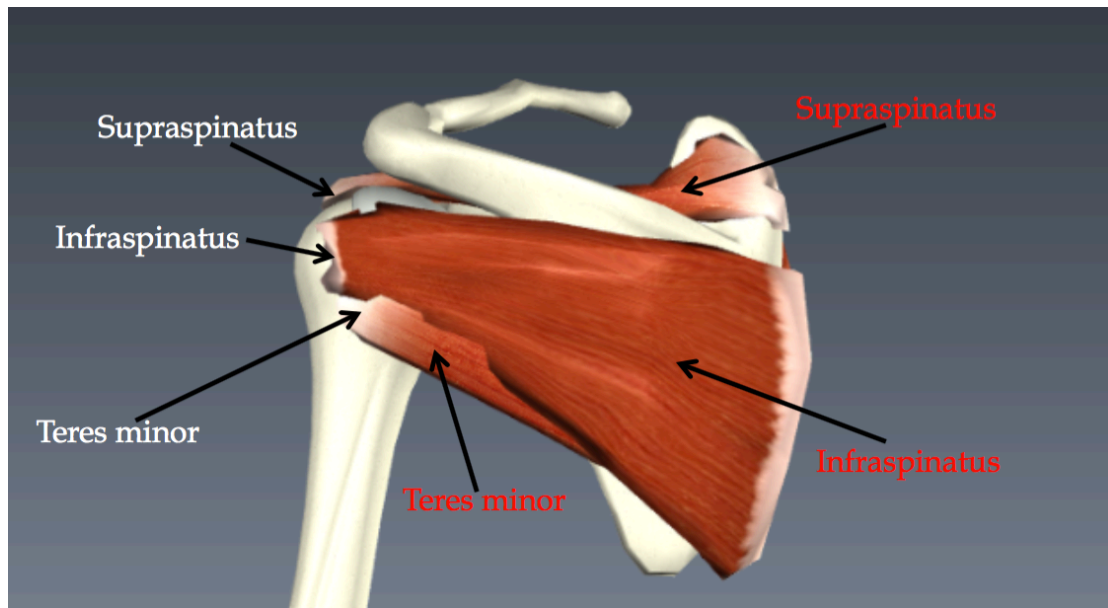


Figure 1-4. Rotator muscles and tendons of the left shoulder (posterior view).

Image was taken from www.biodigital.com (2). Muscles are labelled in red and tendons are labelled in white.

1.1.1.1 Rotator cuff biomechanics

The muscles of the rotator cuff help move the shoulder in abduction, internal rotation, and external rotation. However, the muscles do not contribute equally to movement. Approximately 53% of shoulder movement is controlled by the subscapularis, 20% from the infraspinatus, 14% from the supraspinatus, and 10% from the teres minor (3). While their relative contributions to movement vary, the rotator cuff muscles act together in force couples to maintain stability throughout the shoulder's range of movement (4).

The shoulder experiences complex forces in shear, torsion, compression, and tension that can reach 100MPa during vigorous exercise (5). However, like many tendons, the rotator cuff tendons are most commonly

loaded in tension (6). When loaded in tension, tendons follow a characteristic stress-strain curve (figure 1-5). Stress on an object is equal to the force applied (F) divided by the area of the object (A) through which the force is applied. Strain is equal to the change in length of the object (ΔL) divided by the original length of the object (L_0). The staggered collagen molecules in the tendon create a 'crimp' pattern, which straightens under low strain ($\approx 2\%$) and creates the 'toe region' on the graph (figure 1-5) (7). This feature is thought to minimise the damage that could result when tendons are rapidly loaded (8). Tendon strains up to 4% fall within the physiological range because up to this limit, collagen fibres can recoil and assume their original form without damage. However, at strains above 4%, microscale tearing and slippage between the collagen fibres occur, which are not reversible (7). Macroscopic failure of the tendon occurs at strains above 8%.

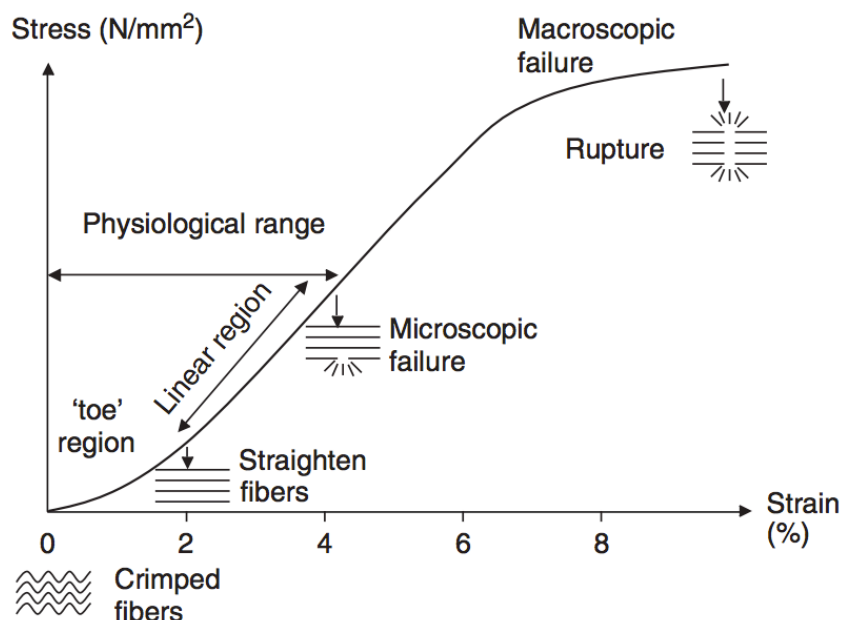


Figure 1-5. Representative stress/strain curve for tendon.

Image taken from Wang *et al.* (7).

However, healthy tendons can withstand very high forces before they reach the point of macroscopic failure. Under tension, an intact supraspinatus tendon fails at $1978 \pm 301\text{N}$ (9). In fact, it appears that the supraspinatus tendon will detach from the insertion before the tendon itself fails, with detachment forces reported between $652\text{--}784\text{N}$ (10, 11). The supraspinatus tendon stiffness appears to be $81\text{--}130\text{N/mm}$ (12) and is able to retain sutures up to forces of $168\text{--}228\text{N}$ (13). It also has an ultimate stress of $4\text{--}16\text{MPa}$ and modulus of elasticity of $50\text{--}180\text{N/mm}^2$ (10).

1.1.2 Microscopic structure of the rotator cuff

Rotator cuff tendons are complex, hierarchical structures with microscopic structures that have evolved to meet the demands of the shoulder joint.

1.1.2.1 Enthesis

The enthesis is the structure that connects rotator cuff tendons to the head of the humerus. It is composed of four distinct phases, with each phase constituting different types of tissue (figure 1-6). The tendon is composed mainly from type I collagen and the cellular components are tenocytes (14). The tendon is connected to uncalcified fibrocartilage that contains type II and type III collagen, and chondrocytic-type cells (14). This then switches to calcified fibrocartilage that also contains chondrocytes (14), which subsequently connects to the bone of the humeral head, which is populated mainly by osteocytes (14). These transitional zones enable the effective transmission of force while avoiding stress concentration (15, 16).

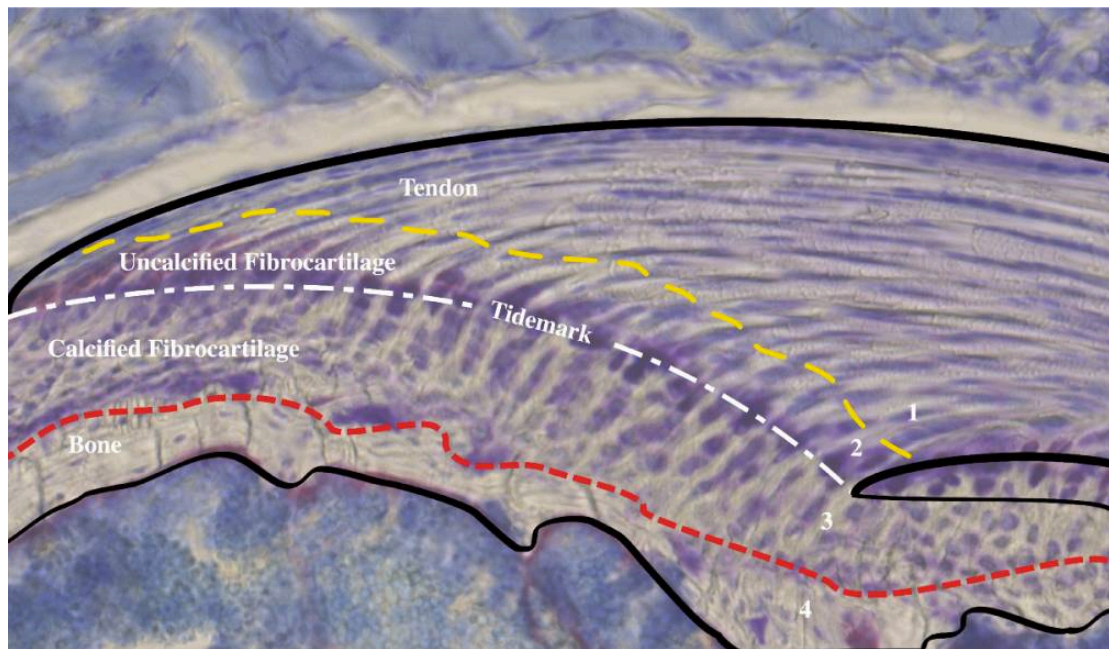


Figure 1-6. Representation of the four zones of the enthesis.

Image is overlaid upon a histological section of the enthesis of a mouse supraspinatus tendon. Image taken from Apostolakos *et al.* (17). Section was stained with toluidine blue to highlight proteoglycan content.

Rotator cuff tendon structure varies not only along the length, but also along the width and depth of the tendon (18) (figure 1-7). In layer 2 (figure 1-7), parallel arrays of tendon fascicles stretch along the length of the supraspinatus and infraspinatus tendons (18), which is an adaptation to tensile loading. In the deeper layer 3, the tendon fascicles are smaller and cross at 45 degrees as they approach the insertion (18), which is thought to be the result of shear forces applied to the tendons. The anterior portion of the supraspinatus tendon is the thickest portion (19, 20), which can help explain why anterior supraspinatus tears are rare.

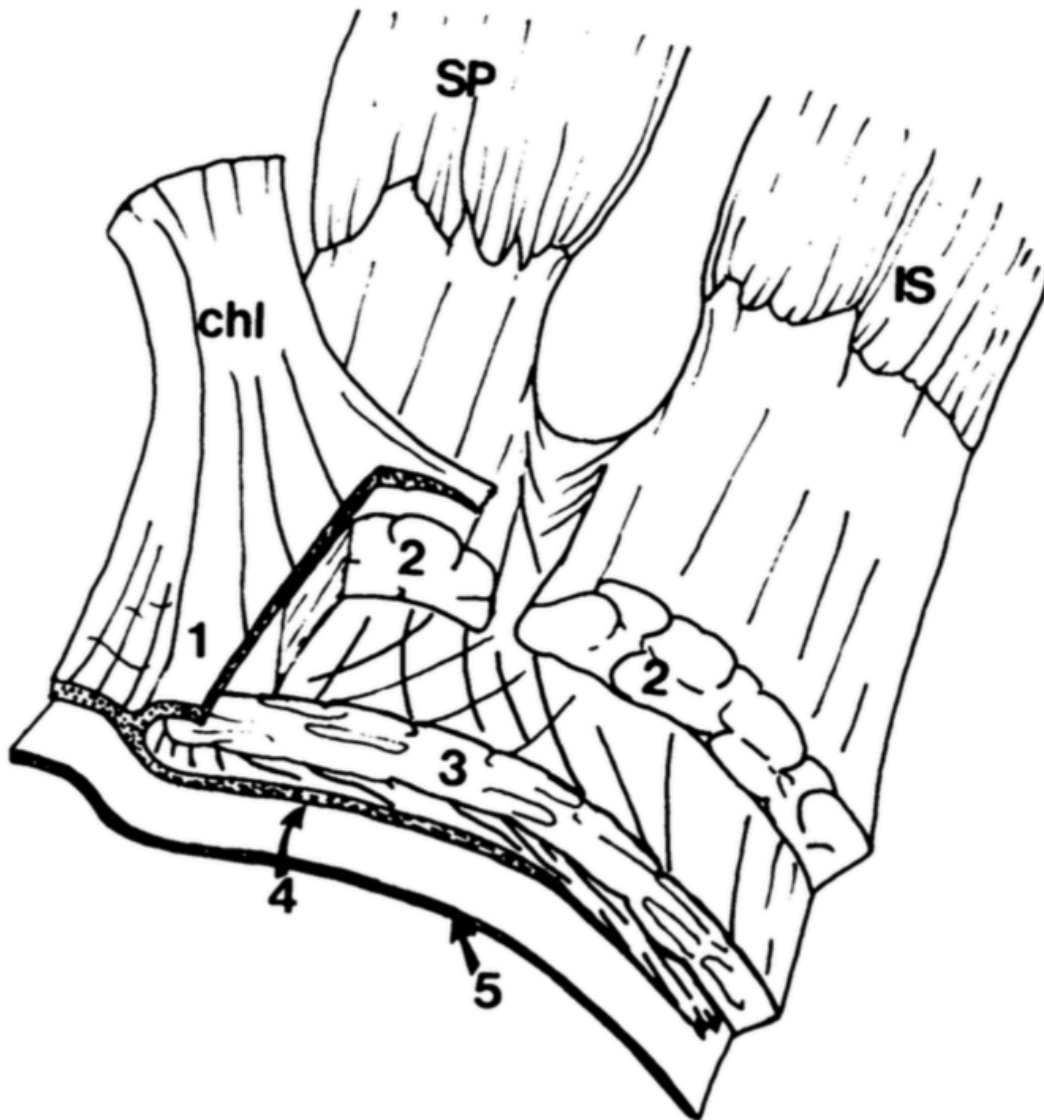


Figure 1-7. Superficial and deep layers of the rotator cuff tendons.

Image taken from Clark & Harryman (18). SP=supraspinatus, IS=infraspinatus. Layers labelled from superficial to deep 1-5.

1.1.2.2 Extracellular matrix

The extracellular matrix (ECM) is the non-cellular component of all tissues.

The ECM is fundamentally composed of water, proteins, and polysaccharides, but the precise composition depends upon the tissue (21). It provides a

scaffold for cell attachment, while offering biophysical and biochemical cues to direct cell behaviour (21). The ECM is dynamic and is continuously being synthesized, remodelled, and degraded (22).

1.1.2.2.1 *Collagens*

Collagen is the fundamental building block of tendon ECM. Collagen accounts for 65-80% of the dry mass of tendon (23), with collagen type I accounting for 95% of all collagen in tendon (24). Type III collagen accounts for 3% of collagen in normal tendon and is mainly found in the endotenon and epitenon (25). Collagen types IV, V, and VI are also present in tendons, but at much lower levels (26).

The building of collagen molecules follows a highly ordered sequence. Following post-translational modifications, three collagen proteins coalesce to form the collagen triple helix. All types of collagen form triple helices, but are distinguished by different amino acid sequences within the collagen proteins. After exocytosis, N-terminal and C-terminal propeptides are removed from the collagen triple helix, creating *tropocollagen* (figure 1-8). Adjacent tropocollagen molecules polymerize together in a staggered formation, 67nm apart, to form collagen fibrils (figure 1-8). This creates the characteristic crimp pattern, which enables the tendon to elongate under tension and create the toe region of the stress-strain curve (figure 1-5)(27, 28). Even though fibrils are often longitudinally aligned, they can form cross, spiral, and plait formations depending upon the forces the tendon experiences (29).

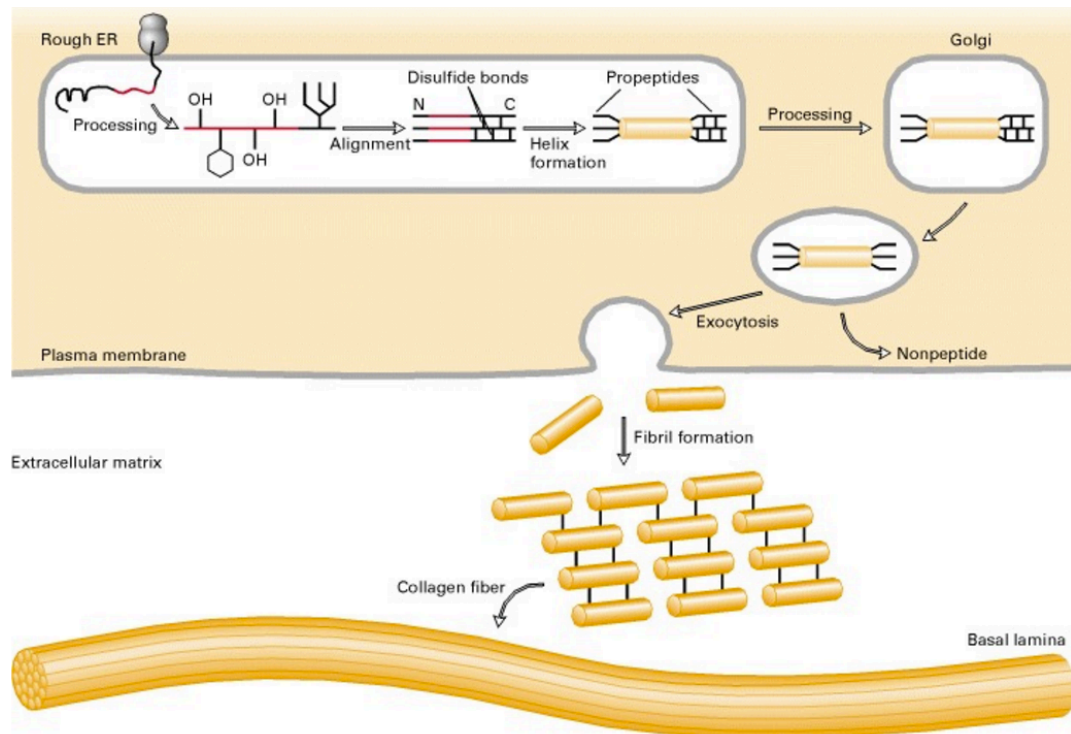


Figure 1-8. Steps during the collagen synthesis and tendon assembly.

Image taken from Lodish *et al.* (30).

Collagen fibrils progressively bundle together to form collagen fibres that range from 1-20 μ m in diameter (figure 1-9). Several collagen fibres collect together to form a primary fibre bundle (subfascicle), which collect together to form a secondary fibre bundle (fascicle), and these further collect together to form tertiary fibre bundles (figure 1-9). Tertiary fibre bundles collect together to form a tendon unit and these subsequently group together to form the tendon (figure 1-9).

A fine layer of connective tissue known as the endotenon separates the primary, secondary, and tertiary fibre bundles (figure 1-9). Each tendon unit

and the tendon itself is surrounded by the epitenon, which contains the nerve, lymphatic, and vascular supply of the tendon (28). Some tendons, like those in the hand, are enclosed within a fibrous sheath that protects the tendons during movement. Between the tendon and the sheath is a loosely-associated fibrous tissue called the paratenon (7). While rotator cuff tendons are not enclosed within sheaths, they do have a paratenon. The outer epitenon and paratenon make the peritendon, which helps to reduce friction between rotator cuff tendons and adjacent tissues in the shoulder (31).

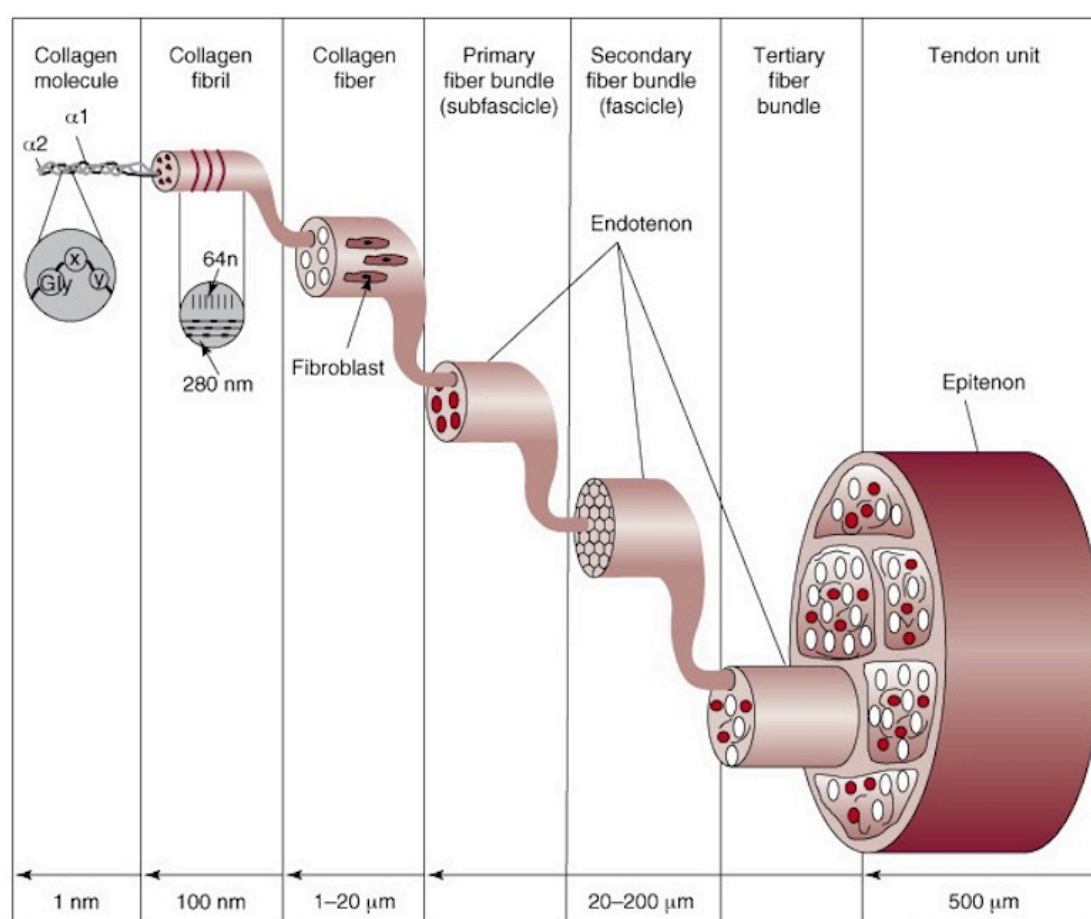


Figure 1-9. Hierarchical structure of tendon.

Originally published by Silver *et al.* (32), but an adapted version was taken from <http://www.thoroughbredhealthnetwork.co.uk/wp-content/uploads/2015/11/Tendon-fibril-structure2.jpg> (33).

1.1.2.2.2 *Proteoglycans and glycosaminoglycans*

Proteoglycans are heavily glycosylated proteins found in the ECM. They consist of a core protein with one or more attached glycosaminoglycan (GAG) side chains (figure 1-10) (21). GAGs are unbranched polysaccharide chains composed of repeating disaccharide units (21). The most abundant GAGs within tendon are hyaluronic sulphates, dermatan sulphate, chondroitin 4 sulphates, and chondroitin 6 sulphates (34). GAGs are extremely hydrophilic and trap water to give tendons specific viscoelastic properties (properties of both a viscous substance and an elastic substance). The water content enables tendons to resist compression and allows collagen fibres to glide past each other more easily (35).

Proteoglycans link collagen fibrils together and actively participate in fibril formation (35). There are a variety of proteoglycans, including decorin, biglycan, aggrecan, versican, fibromodulin, and lumican (35). Proteoglycans such as decorin, lumican, and fibromodulin play an important role in guiding and stabilizing collagen fibril formation and maturation (36). Rotator cuff tendons have increased GAG content relative to other tendons, suggesting that rotator cuff tendons experience more compressive forces (34).

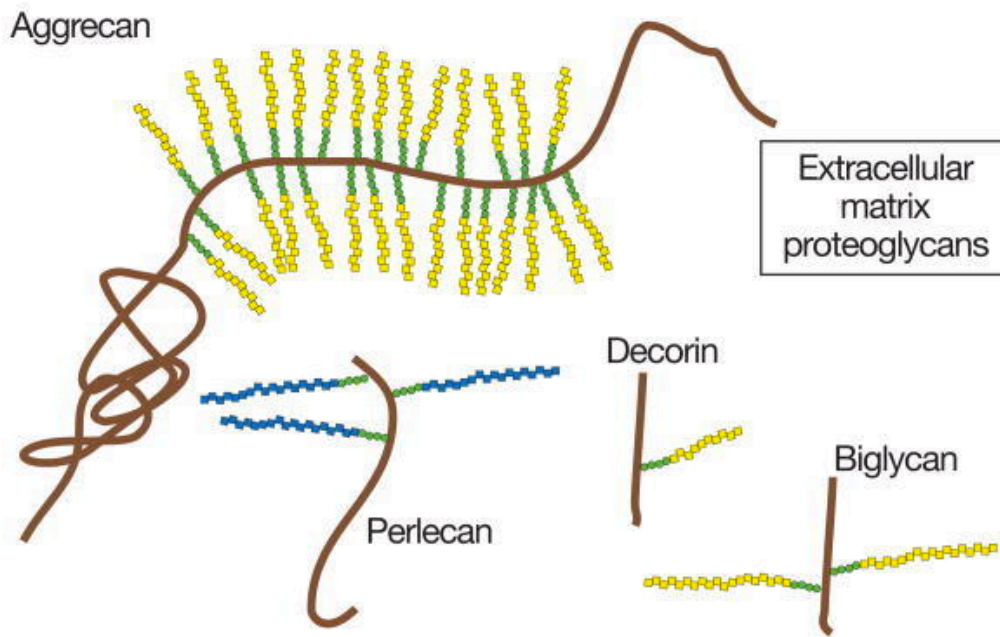


Figure 1-10. The structure of proteoglycans.

Aggrecan (upper) with examples of glycosaminoglycans (GAGs) perlecan, decorin, and biglycan (lower). Image taken from Esko *et al.* (37). The core protein is represented in brown and the GAGs are represented in blue and yellow. Connecting proteins are represented in green.

1.1.2.2.3 Elastin

Elastin is approximately 1-2% of tendon by mass. It is a non-glycosylated protein that is extensively cross-linked to enable recoil following tendon loading (38).

1.1.2.3 Cells

1.1.2.3.1 Tenocytes

The primary cell type in tendons is the tenocyte, which is a mesenchyme-derived fibroblastic cell. Tenocytes produce and organize tendon-specific ECM components, which provide the tendons with their mechanical

properties (39). The type and amount of ECM components that tenocytes produce are dependent upon the environmental signals such as surface roughness (40), stiffness (41), topography (42), mechanical loading (7), and growth factor presence (43) (figure 1-10).

Rows of tenocytes are sparsely distributed throughout the tendon (44). Tenocytes from healthy tendon tissue demonstrate an elongated bi-directional morphology, whereas diseased or apoptotic tenocytes tend to present with a more rounded morphology (45, 46). Many tenocytes are connected via gap-junctions, enabling the efficient communication of received signals to other cells (44, 47). Tenocytes can generally be identified by gene expression of *COL1A2*^{high} and *SCX*^{high} and *TNC*^{low}, but no specific marker for tenocytes currently exists (48). Tenocytes have limited differentiation potential (49) and low mitogenic activity (50), the latter point has been suggested as one of the reasons why tendons take so long to heal (51).

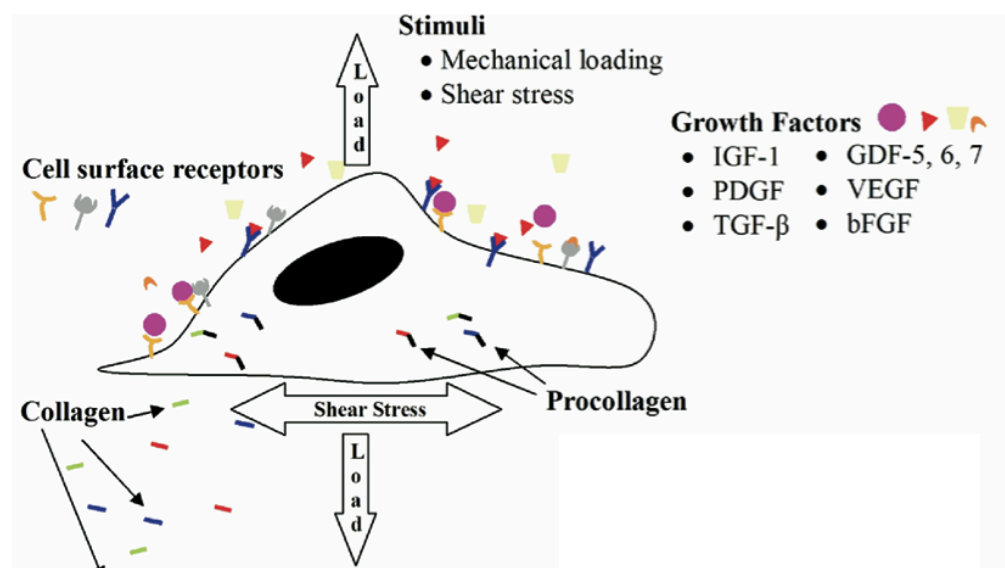


Figure 1-11. Schematic of tenocyte response to various stimuli.

Image taken from James *et al.* (52).

1.1.2.3.2 Resident macrophages

The subacromial bursa is thought to contain both type A and type B synoviocytes, with the former considered a type of resident macrophage and the latter a fibroblastic cell capable of synthesizing ECM components (53). Tendons contain resident anabolic macrophages, which accumulate at the site of tendon injury after several days (54, 55). Macrophage infiltration leads to an increase in the production of TGF- β , which is thought to be associated with increased fibrovascular scar formation in the healing tendon (56, 57).

While macrophage infiltration is a common process following the injury of many tissues, macrophages can accumulate and stay at the site of tendon injury. A rat rotator cuff model used by Gumucio *et al.* (55) showed that macrophages were present in large numbers at the repair site even 30

days after the initial injury. Such macrophages have been specifically associated with fatty deposits (58) and are thought to be similar to the macrophages that promote inflammation and fatty deposition in atherosclerosis (59). Indeed, Gumucio *et al.* (60) suggest that such macrophages may play an important role in the fatty infiltration seen in rotator cuff tears.

1.2 Rotator cuff tendinopathy

Rotator cuff tendinopathy, or rotator cuff disease, a term generally used to describe different pathologies associated with the pain and dysfunction of the rotator cuff and its surrounding structures (61). It is a generalised term that encompasses a spectrum of different ailments that includes tendon inflammation, tendon degeneration, calcific tendinopathy, glenohumeral arthritis, and tears of the rotator cuff tendons (62).

1.2.1 Rotator cuff tears

A rotator cuff tear occurs when the fibres of the tendon are broken. Tears can occur in any of the rotator cuff tendons, but tears in the supraspinatus tendon are most common (33).

1.2.2 Prevalence of rotator cuff tears

Rotator cuff tears are a common clinical problem. In the US, they cause 4.5 million physician visits each year (63) and 17 million people are at risk of disability from rotator cuff failure (64). In the UK, 14% of the population report experiencing shoulder dysfunction, and rotator cuff tendinopathy disorders are among the most common causes of pain (65, 66). Prevalence in the general population has been reported between 5% (67) and 59% (68) with an average of 24% (67-75). Partial thickness tears only extend partially through the depth of the tendon, whereas full thickness tears extend through the entire depth. Partial thickness tears are more common than full thickness tears (69, 75) and the prevalence of all types of tears increases with age (68-74, 76), with tears seen in 6% under 60 compared to 30% over 60 years of age (71).

Approximately 20% of the US population will be 65 years or older by 2030 (77), so the number of patients with rotator cuff tears will likely increase.

1.2.3 Socioeconomic burden of rotator cuff tears

Shoulder pain caused by rotator cuff tears, can impede activities of daily living (78-80), lead to time off work (81, 82), and even unemployment (83). Conservative treatments such as glucocorticoid injections (84) and physiotherapy (85) may provide symptomatic relief in the short term. However, large tears do not heal spontaneously and often require surgical intervention (86). Between 75,000 and 300,000 rotator cuff surgeries are performed in the USA (87-90) and 10,000 are performed in the UK each year (91). The number of surgeries being performed is increasing in the US (90) and Europe (91, 92). Most surgeries are now performed arthroscopically (93-96), but more experienced surgeons still prefer open or mini-open procedures (93). The cost of rotator cuff surgery is approximately \$13,000 per patient (97), placing an annual burden of \$3 billion on the US (89) and \$250 million on Australia (98). In 2000, treatment of shoulder dysfunction cost the United States \$7 billion (99). Despite the large financial burden, rotator cuff surgery is cost effective (87, 97) with an age-weighted societal saving of \$13,771 over a patient's lifetime and estimated annual societal savings of \$3.44B in the United States (97).

1.2.4 Aetiology of rotator cuff tears

Several factors have been associated with the development and progression of rotator cuff tears.

1.2.4.1 Anatomical variation

Charles Neer proposed that up to 95% of rotator cuff tears were caused by the impingement of the coracoacromial arch upon the supraspinatus tendon (67). This theory gained prominence when Louis Bigliani demonstrated that acromial shape correlated with rotator cuff tears (100). Bigliani found that 70% of patients with hooked or curved acromions had rotator cuff tears, while only 3% of patients with flat acromions presented with tears (100). Furthermore, acromion anatomy changed from flat to curved and hooked, as the patients aged (101). However, this theory cannot fully explain why most partial thickness tears are intra-tendon or on the articular side (102, 103), not the bursal side where the acromion is located.

1.2.4.2 Genetics

Several studies have suggested that genetics plays a role in the development of rotator cuff tears. Harvie *et al.* (104) showed that the siblings of people with full-thickness rotator cuff tears are 2.42 times more likely to also have a full-thickness rotator cuff tear, compared to their spouses (controls). The relative risk of a full-thickness symptomatic tear in siblings compared to controls was even higher at 4.65 (104). Tashjian *et al.* (105) showed that first, second, and third degree relatives of people with rotator cuff disease are significantly more likely to also have rotator cuff disease, compared to unrelated controls.

Genetics have also been implicated, not just in the development, but also in the progression of rotator cuff tears. Gwilym *et al.* (106) showed that rotator cuff tears are significantly more likely to increase in size if the patient has a sibling with a rotator cuff tear, compared to a patient without a sibling

that has a torn rotator cuff tendon. Even though a genetic polymorphism has been associated with Achilles tendinopathy (107), currently no alleles or polymorphisms have been directly associated with an increased risk of rotator cuff pathology (108).

1.2.4.3 Age

Rotator cuff tears disproportionately affect older patients, with 31% of patients over the age of 70 presenting with a rotator cuff tear (109). Fehring *et al.* (76) demonstrated a 2.69 fold increase in rotator cuff tear prevalence with every 10-years above 65 years of age and Nobuhara *et al.* (110) found 97% of rotator cuff surgeries are performed on patients over the age of 45. However, some elderly patients never develop rotator cuff tears, so other factors must also contribute.

1.2.4.4 Overuse

The overuse theory rests on the premise that repetitive loading at submaximal forces causes micro-damage in the tendon, which is not fully repaired and subsequently accumulates over time (111). Matrix metalloproteinases (MMPs) degrade ECM components, and increased MMP expression has been observed in torn supraspinatus tendons compared to controls (112). Overuse may also increase the presence of oxygen free radicals and apoptotic mediators, which can trigger apoptosis in tendon cells and subsequently reduce ECM production (113). Overuse has been shown to cause tendon tears in animal models (114) and can help explain the prevalence of rotator cuff tears in dominant arms (72, 115). However, this theory alone does not explain why rotator cuff tears develop in sedentary patients (116).

1.2.4.5 Vascularity

Some studies have suggested a 'critical zone' approximately 1cm from the insertion of the supraspinatus where vascularity is greatly reduced (117). It has been postulated that the hypovascular area is more susceptible to degenerative changes and thus at greater risk of tear development. However, studies using Doppler flowmetry (118) and histological analysis of torn rotator cuff tendons (119) have actually shown increased vascularity in the 'critical zone.'

1.2.4.6 Inflammation

Inflammation has been implicated in the development of rotator cuff tears. Inflammatory cells are more abundant in torn tendons (57), and inflammatory genes and proteins are highly expressed in overused tendons (120). The amount of inflammation is inversely correlated with tear size (121). Kannus & Jozsa (122) suggest that inflammation occurs early in the development of rotator cuff pathology and is largely absent by the time rotator cuff tears are large and chronically degenerate.

The source of this inflammation remains unclear, but several studies have identified elevated inflammation in tissues adjacent to the rotator cuff tendons. Sakai *et al.* (123) found increased levels of TNF- α , IL-1, TGF- β , and FGF in the subacromial bursa of patients with rotator cuff tears compared to patients with shoulder instability, but no rotator cuff tear. Similar results were found by Voloshin *et al.* (124). Abrams *et al.* (125) also reported elevated levels of inflammation in the synovium of patients with rotator tears compared to those without rotator cuff pathology.

More recently, studies have shown that tenocytes increase the expression inflammatory genes in response to stimuli such as lipopolysaccharide (LPS) (126) and platelet rich plasma (PRP) (127). These findings show that tenocytes not only respond to, but also contribute to inflammation. Collectively, these studies suggest that inflammation plays a greater role in the development of rotator cuff tears than was previously appreciated.

1.3 Rotator cuff repair

1.3.1 Natural stages of rotator cuff repair

When the rotator cuff tendons become injured, the body mounts a sequential healing response in an attempt to restore structure and function. This involves three distinct stages: inflammation, proliferation, and remodelling. These stages often overlap and can vary in length of time depending upon the patient. The natural repair mechanisms may be sufficient in smaller tears, but larger tears often require surgical repair.

1.3.1.1 Inflammatory stage

The inflammatory stage begins within seven days of the injury. Formation of a hematoma at the injury site releases chemotactic factors such as Insulin-like growth factor 1 (IGF-1), platelet derived growth factor (PDGF), and transforming growth factor-beta (TGF- β), which attract inflammatory cells to the injury site. Neutrophils and macrophages phagocytose debris and recruited tenocytes synthesize ECM components (128). Recruited macrophages secrete TGF- β which contributes to scar tissue formation (56).

1.3.1.2 Proliferative stage

The proliferative stage involves the recruitment and proliferation of tenocytes in an effort to increase ECM production. At this stage, the ECM is composed largely of type III collagen and proteoglycans, which form a physical continuity at the injury site. Bone morphogenic protein-12 (BMP-12) and bone morphogenic protein-14 (BMP-14) are thought to be important components of the healing process and Seeherman *et al.* (129) demonstrated superior repair

by delivery of exogenous BMP-12 into a full thickness rotator cuff sheep model. IGF-1 (130) and basic Fibroblast growth factor (bFGF) (131, 132) have also been implicated as important regulators of cell behaviour during the proliferative stage. During this stage, new nerves and blood vessels develop around the injury site, connecting it with existing tissue.

The healing tendon responds to mechanical loading and a delicate balance must be struck during the proliferative stage; too little mechanical loading causes scar tissue to develop and form deleterious adhesions that restrict the gliding of the tendon. Too much mechanical loading can excessively stress the weakened tendon and can lead to re-injury.

1.3.1.3 Remodelling Stage

Approximately 6-8 weeks after injury, the repairing tissue enters the remodelling stage. This stage is characterized by a reduction in cellularity and an increase in type I collagen and matrix metalloproteinases (MMPs). MMPs are a family of at least 24 zinc-dependent endopeptidases that are responsible for the degradation of ECM protein components (133). MMPs facilitate the turnover of tendon ECM and are upregulated when the tendon is mechanically loaded (134, 135). Tissue inhibitors of matrix metalloproteinases (TIMPs) inhibit MMP activity and restrict the degradation of the ECM to strike a healthy balance that maintains the integrity of the ECM.

IGF-1 and TGF- β are also expressed during the remodelling stage and are thought to contribute to scar formation (52, 136). ECM components become more organized in an attempt to recreate the original tendon tissue.

However, the body is never able to fully reconstruct the complex enthesis that was originally present.

1.3.2 Conservative treatments

Almost every patient is initially treated conservatively, with the exception of acute traumatic tears that may benefit from immediate surgery (137). Regardless of tear size, many patients can improve function and reduce pain by using a combination of conservative treatments (138).

1.3.2.1 Glucocorticoid injection

Glucocorticoid steroid injections are a common short-term treatment that provide pain relief (84). While a single steroid injection does not significantly affect the composition of the native tendon (139), repeated steroid injections have been associated with diminished cellular proliferation and tissue degeneration (140, 141). Steroid injections have also been implicated in the rupture of weight-bearing tendons (142, 143).

1.3.2.2 Physiotherapy

Physiotherapy is a common conservative treatment for rotator cuff tears, but is also used during post-operative rehabilitation. Itoi & Tabata (85) demonstrated an 82% success rate using physiotherapy alone to treat rotator cuff tears with an average follow-up of 3.4 years. A similar study by Bartolozzi *et al.* (144) reported a 66% success rate in treating patients with rotator cuff tendinopathy and impingement. However, unfavourable outcomes were associated with patients who had larger tears (>1cm) and symptoms that lasted more than 1 year (144).

1.3.3 Surgical treatments

Surgical treatments are reserved for patients who have not clinically improved following conservative treatments. While surgery can reduce pain and improve function in many patients, surgery-associated complications such as infection, scarring, and nerve damage can detrimentally affect the clinical outcome.

1.3.3.1 Subacromial decompression

Subacromial decompression (SAD) is one of the most common surgical procedures performed for rotator cuff tears. The procedure involves burring the underside of the acromion to reduce impingement upon the supraspinatus tendon. Several studies have demonstrated that SAD can reduce pain and improve function (145, 146). However, a systematic review by Brox *et al.* (147) found no significant difference in outcomes for SAD in comparison with physiotherapy alone in patients with rotator cuff tendinopathy. It has also been suggested that SAD causes glenohumeral instability, which may lead to tear propagation (148).

1.3.3.2 Rotator cuff repair

Rotator cuff repair surgery is considered the final therapeutic option for patients. Uncertainty remains regarding the features that should guide the decision to operate. The surgery involves suturing of the torn edges of the tendon back together and trying to restore the native rotator cuff footprint. Rotator cuff repair can be performed via an open, mini-open or arthroscopic repair both with and without an accompanying SAD and using a variety of

suturing techniques. However, there is little evidence to demonstrate the superiority of one technique over another (149).

1.3.4 Re-tear rate

Developments in surgical techniques have continued to improve the strength and stability of repaired tendons (150). However, re-tear rates following surgical rotator cuff repair have been reported between 11% (151) and 94% (152) with an average of 39% (152-164). Factors associated with repair failure include tear size (144, 160, 165-168), age (154, 158, 165, 169), magnitude of fatty infiltration (170), quality of tendon tissue (162, 166, 169, 171), repair technique (130, 151, 153, 172), previous failed surgical repair (173), postoperative rehabilitation protocol (166), smoking (174) and comorbidities such as diabetes (175). It is clear that an effective clinical solution to reduce re-tear rates is required.

1.4 Scaffolds

In an effort to reduce re-tear rates, many shoulder surgeons are using scaffolds to augment rotator cuff repairs. However, with more than 20 different scaffolds currently available, questions regarding their mechanical properties, potential to modulate native tenocyte behaviour, immunogenicity, and clinical efficacy remain.

1.4.1 What are scaffolds?

Scaffolds are implantable biomaterials used in surgery to support or replace tissue where weakness exists. Scaffolds provide mechanical support and can, in theory, enhance native biological healing processes. Over 20 scaffolds are currently available to augment rotator cuff tears, the characteristics of which, including size, shape, material, porosity, strength, thickness, and stiffness can vary considerably.

There are three main types of scaffolds: biological, synthetic, and biosynthetic. Some examples of different commercially available scaffolds are presented in table 1-1. Each scaffold type will be briefly discussed in the following sections.

Introduction

Table 1-1. Table of commercially available scaffolds.

Scaffold Name	Manufacturer	Material	Type	Cross-linked
TissueMend	TEI Biosciences, Inc	Bovine dermis	Biological	NO
GraftJacket	LifeCell Corporation	Human dermis	Biological	NO
ArthroFlex	Arthrex	Human dermis	Biological	YES
AlloPatch HD	Musculoskeletal Transplant Foundation	Human dermis	Biological	NO
Conexa	Tornier	Porcine dermis	Biological	NO
Medeor Matrix	DSM Biomedical	Porcine dermis	Biological	NO
Zimmer Collagen Repair Patch (formerly Permacol)	Tissue Science Laboratories Plc	Porcine dermis	Biological	YES
Fascia Lata	Musculoskeletal Transplant Foundation	Human fascia lata	Biological	NO
Permacol	Covidien	Porcine dermis	Biological	YES
Sportmesh	Artelon	Polyurethane urea	Synthetic	NO
X-repair	Synthasome	Poly- L-lactic acid	Synthetic	NO
Soft Tissue Regeneration Graft	Soft Tissue Regeneration	Poly-lactic acid	Synthetic	NO
BioFiber	Tornier	Poly(4-hydroxybutyrate)	Synthetic	NO
Biomerix RCR Patch	Biomerix	Polycarbonate poly(urethaneurea)	Synthetic	NO
LARS ligament	LARS	Polyethylene terephthalate	Synthetic	NO
Poly-tape	Neoligaments	Polyethylene terephthalate	Synthetic	NO
Leeds-Kuff Patch	Neoligaments	Polyethylene terephthalate	Synthetic	NO
BioFiber CM	Tornier	Poly(4-hydroxybutyrate) + bovine collagen	Biosynthetic	NO

1.4.2 Biological scaffolds

Biological scaffolds are derived from mammalian tissue, either from humans (allografts) or other mammalian species (xenografts).

1.4.2.1 Manufacturing

Biological scaffolds are often processed to remove native cells, lipids, and polysaccharides, but leave the native ECM intact. These steps are performed to reduce antigenicity and to minimise the chance of disease transmission. A balance must be struck between the removal of harmful antigens and the preservation of the ECM architecture.

1.4.2.1.1 Decellularisation

Cellular components are removed from biological scaffolds using mechanical, enzymatic, and chemical techniques. Common decellularisation techniques include rinsing in hypertonic salt solutions, detergents, and enzymatic solutions, as well as snap freezing (176).

1.4.2.1.2 Cross-linking

Some biological scaffolds are artificially cross-linked to increase mechanical properties (177), slow degradation (178), and mask antigenic epitopes (176). Various cross-linking agents such as glutaraldehyde, carbodiimide, genipin, proanthocyanidin, isocyanate, and polyepoxy compound have been used (179).

1.4.2.1.3 Lyophilisation

Biological scaffolds can be lyophilised (freeze-dried) to preserve the tissue architecture and extend shelf life. Freytes *et al.* (180) found that lyophilised porcine urinary bladder was thinner and increased fibroblast proliferation more compared to a non-lyophilised control. Roy & Gupta (181) found that freeze-drying induces conformational changes in native growth factors, which may influence bioactivity.

1.4.2.1.4 Sterilisation

Many biological scaffolds are terminally sterilized to reduce the presence of harmful pathogens, such as bacteria and viruses. Techniques that are used include ethylene oxide gas, gamma radiation, and electron beam radiation. Allografts are the exception to this, as they are frequently treated with antibiotics and aseptically processed without additional sterilisation. While terminal sterilisation reduces the presence of harmful pathogens, it can unintentionally alter the scaffold. The mechanical properties of porcine urinary bladder ECM treated with ethylene oxide, gamma radiation, or electron-beam radiation were significantly reduced compared to the non-sterilized control (182). Grimes *et al.* (183) also showed that irradiated porcine small intestine submucosa (SIS) degraded more rapidly in solution compared to a non-sterilized control.

1.4.2.2 Composition

1.4.2.2.1 ECM proteins

As biological scaffolds are made from ECM, their characteristics are determined by the species, age, and health of the donor animal, in addition to

site of the source tissue. While biological scaffolds can, in theory, be derived from any tissue, they are most commonly made from dermis, fascia lata, pericardium, and small intestine submucosa (SIS). This means that they are predominantly made of type I collagen (184), but can also contain smaller amounts collagen types III, IV, V, and VI (185). Glycosaminoglycans (GAGs) such as heparan sulphate and hyaluronic acid are also present (186), in addition to adhesion molecules like laminin (187) and fibronectin (188).

1.4.2.2.2 Growth factors

Biological scaffolds can retain growth factors such as vascular endothelial growth factor (VEGF) (189), basic fibroblast growth factor (bFGF) (190), and transforming growth factor-beta (TGF- β) (190, 191). Many of these factors retain their biological activity even after harsh processing techniques (191, 192).

1.4.3 Synthetic scaffolds

Synthetic scaffolds are made from either absorbable or non-absorbable polymers.

1.4.3.1 Manufacturing

Synthetic scaffolds can be manufactured using a variety of techniques. Popular methods include using heat, pressure, and catalysis to polymerise monomers into polymeric chains. Polymers can be further processed using techniques such as extrusion or electrospinning to change the physical structure of the scaffold. Each step during the manufacturing process can be precisely controlled, so that uniform products can be produced. Synthetic

scaffolds are sterilized using gas or radiation, but high doses of radiation can also introduce unwanted cross-links (193).

1.4.3.2 Composition

Absorbable synthetic scaffolds are constructed from biodegradable polymers such as polyglycolic acid (PGA) and polylactic acid (PLA). These can provide initial mechanical support and will degrade over time into natural metabolites that can be processed and excreted by the body. In contrast, non-absorbable synthetic scaffolds are made from polymers such as polyethylene terephthalate (PET), which takes years or even decades to degrade.

Synthetic scaffolds can be made from a variety of different polymers and assume a variety of different forms. Synthetic fibres can be different diameters and lengths, and arrayed into a multitude of different woven or knitted patterns.

1.4.4 Biosynthetic scaffolds

Biosynthetic scaffolds are most commonly synthetic scaffolds coated in biological substances, such as collagen.

1.4.4.1 Manufacturing

The synthetic component is often made in the same way as synthetic scaffolds are, with processes such as extrusion and weaving. The biological components are often attached using proprietary techniques.

1.4.4.2 Composition

The composition of biosynthetic scaffolds can vary significantly. Currently, two commercially available biosynthetic scaffolds exist. Tornier's BioFiber-

CM is made from woven fibres of poly-4-hydroxybutyrate (PH4B) that are coated with bovine collagen. PH4B is an absorbable polymer, so the body would eventually absorb BioFiber-CM. OrthADAPT PR is another commercially available example, which is made from woven polyetheretherketone fibres coated with equine pericardium ECM. Despite the theoretical benefit of biosynthetic scaffolds, few studies have extensively evaluated their performance.

1.5 Mechanical properties of scaffolds

1.5.1 Macroscale mechanical properties of scaffolds

Macroscale mechanical properties encompass the bulk material properties of a scaffold. These properties include tensile strength, suture-retention, stiffness, loss-modulus (the relative ability to lose energy), and storage modulus (the relative ability to store energy). These properties are determined by the material, structure, and orientation of the scaffold.

1.5.1.1 Mechanical testing of scaffolds alone

Several studies have examined the macroscale mechanical properties of different scaffolds (194-199). Barber *et al.* (194) evaluated the mechanical properties of Allopatch HD1, Allopatch HD2, GraftJacket MaxForce, GraftJacket MaxForce Extreme, OrthoAdapt, SportMesh, and rotator cuff allograft. The ultimate strength of the two human dermal scaffolds, (GraftJacket MaxForce and GraftJacket MaxForce Extreme) and two human fascia lata scaffolds (Allopatch HD1 and Allopatch HD2), were greater than the equine pericardium (OrthAdapt) and synthetic (SportMesh) scaffolds. Chaudhury *et al.* (199) also showed that porcine dermis (Zimmer collagen repair patch) and human dermis (GraftJacket) had a higher load of failure than porcine SIS (Restore) and synthetic (SportMesh) scaffolds. The strength of dermal scaffolds may be derived from their significant thickness (195). After controlling for thickness, Derwin *et al.* found that SIS scaffolds were actually stronger than dermal scaffolds (195). However, calculating strength

relative to thickness is less clinically relevant because it is unlikely that surgeons will alter scaffold thickness prior to implantation.

It is noteworthy that biological scaffolds are much more elastic than tendon, with strains at failure reported between 30-80% for dermis (195, 197, 199), 20-35% for SIS (195, 197, 199), and 30-60% for pericardium (197). Tendons can fail at strains above 8% (7), so questions remain regarding the ability of biological scaffolds to provide sufficient mechanical support during the healing process.

Synthetic scaffolds are generally stiffer than biological scaffolds, but several studies have used SportMesh, a particularly elastic synthetic scaffold. Barber *et al.* (194) found that SportMesh stretched the most out of seven scaffolds and Chaudhury *et al.* (199) found that it stretched 82% before failure—more than any other scaffold tested. Despite many synthetic scaffolds existing, few studies have evaluated their mechanical properties.

Processing techniques appear to influence the mechanical properties of biological scaffolds, because studies using different forms of the same tissue have reported differences in mechanical properties (194, 197, 198). Annor *et al.* (197) showed that cross-linked biological scaffolds were most resistant to enzymatic degradation and subsequently maintained their mechanical properties better than non-cross-linked biological scaffolds. However, most processing techniques remain proprietary and their relative influence on scaffold properties remains unclear.

1.5.1.2 Suture retention of scaffolds alone

Surgical rotator cuff repairs most often fail by suture pull out (200) and several studies have evaluated the suture retention properties of commercially available scaffolds (194, 199, 201). Barber *et al.* (201) used a mattress suture technique to test GraftJacket thin, GraftJacket MaxForce, GraftJacket Extreme, Permacol, TissueMend, CuffPatch, and Restore. The dermal scaffolds (GraftJacket thin, GraftJacket MaxForce, GraftJacket Extreme, Permacol, and TissueMend) failed at higher forces than the SIS scaffolds, (CuffPatch, and Restore), with the thicker dermal scaffolds, (GraftJacket MaxForce, GraftJacket Extreme) failing at the highest forces.

Chaudhury *et al.* (199) also found that dermal scaffolds had greater suture retention strengths than SIS and synthetic scaffolds. Even though dermal scaffolds are generally thicker than other biological scaffolds, Barber *et al.* (201) reported that dermal scaffolds (Permacol, and TisseMend) and SIS scaffolds (Restore and CuffPatch) all had thicknesses around 1.1mm. This illustrates the inherent differences in mechanical properties between scaffolds derived from different tissues.

1.5.1.3 Mechanical testing of scaffolds using cadaveric models

Numerous studies have used cadaveric human (12, 202-204) or animal (205) specimens to determine the mechanical properties of scaffold-augmented rotator cuff repairs. In a sheep infraspinatus model, Koh *et al.* (205) found that a synthetic poly-lactic acid scaffold (from Smith and Nephew) increased the force at failure by 1.26 times the non-augmented control. In human cadaveric models, human dermis (GraftJacket) has been shown to increase failure forces

by 1.19 (202) and 1.62 times the control (206). Porcine dermis (Conexa) has also proven to be effective in a human cadaver model, increasing failure forces by 28% (203). However, not all dermal scaffolds are effective. Ely *et al.* (204) found that cross-linked human dermis (Arthroflex) did not significantly increase failure load or reduce gap formation in a human cadaveric model.

X-Repair, an absorbable synthetic scaffold, appears to be very effective at improving the initial mechanical properties of a human cadaveric rotator cuff repair. While augmentation did not increase stiffness, failure loads for superior infraspinatus, anterior supraspinatus, and posterior supraspinatus portions were 72%, 76%, and 56% higher than the controls, respectively. X-Repair augmentation also altered the mode of failure, with only 7/20 augmented repairs failing by the suture pulling through the tendon compared to 17/20 in the non-augmented controls. More of the augmented repairs failed by bony avulsion or rupture in the tendon mid-substance.

1.5.1.4 Mechanical testing of scaffolds using animal models

While cadaveric studies can help evaluate the time-zero mechanical properties of a repair, only animal models can account for changes experienced *in vivo*. Derwin *et al.* (207) used a canine infraspinatus model to test X-Repair and found after 12 weeks, scaffold augmented repairs had less retraction, greater cross-sectional area, greater stiffness, and greater ultimate load compared to non-augmented controls. Santoni *et al.* (208) used a chronic sheep infraspinatus model to test Biomerix RCR mesh (non-absorbable polyurethane mesh). After 3 months, the scaffold-augmented repairs were significantly stronger than non-augmented surgical controls recovering 37.8%

of force of failure and 40.7% of stiffness of intact, non-operated controls. However, not all synthetic scaffolds have demonstrated improvement. MacGillivray *et al.* (209) used an infraspinatus goat model to test a polylactic acid scaffold (Smith & Nephew). At 3, 6, 12, and 24 weeks, there was no difference in the ultimate load to failure between the augmented and non-augmented groups.

The strength of dermal scaffolds seen *in vitro* has also been observed in animal models. In a canine infraspinatus model, Adams *et al.* (210) created a tendon defect that was replaced either with GraftJacket or the original tendon tissue. At time zero both groups demonstrated similar strength, at 6 weeks the GraftJacket-augmented group was actually weaker, but at 12 weeks both groups showed similar strength. The reasoning for the fall in strength at 6 weeks is unclear, but the authors note that GraftJacket could be used to replace tendon tissue, because it had favourable long-term mechanical properties. Ide *et al.* (211) used a rat model to study GraftJacket as an augmentation device. The study compared a GraftJacket-augmented repair to an unrepaired shoulder, so the increased mechanical properties of the GraftJacket group at 2, 6, and 12 weeks were unsurprising.

Dermal scaffolds appear to be slightly stronger in tensile failure than SIS scaffolds in the short term. Using an ovine model, Nicholson *et al.* (212) found that repairs augmented with porcine dermis (Zimmer Collagen Repair Patch) were stronger than porcine SIS (Restore) after 9 weeks. However, no differences were seen between the two groups after 24 weeks. The weakness

of SIS in the short-term has been demonstrated elsewhere. Zalavras *et al.* (213) studied Restore in a rat supraspinatus model to replace a defect, compared to a defect with no replacement. After 6 weeks, no difference in strength between the two groups was observed, indicating the capacity of animal models to spontaneously heal. But, after 16 weeks, the Restore group was stronger than the unrepaired group. Similar results were reported by Dejardin *et al.* (214), who used porcine SIS (Restore) to replace a defect in a canine infraspinatus model. The contralateral shoulder was used as the control and just had the native tendon replace the defect. At 3 and 6 months, the Restore group had similar mechanical properties to the control, with authors suggesting that SIS could be used to replace tissue for large-massive cuff tears. However, SIS is not always effective. Schlegel *et al.* (215) used sheep infraspinatus model with or without porcine SIS augmentation and after 12 weeks, the augmented repairs were stiffer but not stronger than the non-augmented controls.

1.5.1.5 Scaffold orientation and attachment

The way that scaffolds are oriented and attached is an aspect of rotator cuff repair that is often overlooked. Yoder & Elliott (196) evaluated the anisotropy of four commercial scaffolds: Alloderm, Restore, CuffPatch, and OrthADAPT, in linear loading. The study found that Alloderm and OrthAdapt exhibited anisotropy, meaning that the orientation in which these scaffolds are cut significantly influences their mechanical properties.

Three studies have compared different methods of scaffold attachment. Beitzel *et al.* (216) demonstrated that a repair with interposition of a collagen

scaffold or with a dermal scaffold overlay significantly increased ultimate load to failure, compared to a non-augmented control. However, a repair with the interposition of a dermal scaffold or fibrin clot did not improve mechanical properties. A study by Jung *et al.* (217) used a cadaveric sheep infraspinatus model, with a porcine dermal scaffold (DX Reinforcement Matrix patch, Arthrex) augmentation using “integrated”, “cover”, and “hybrid” techniques (figure 1-12).

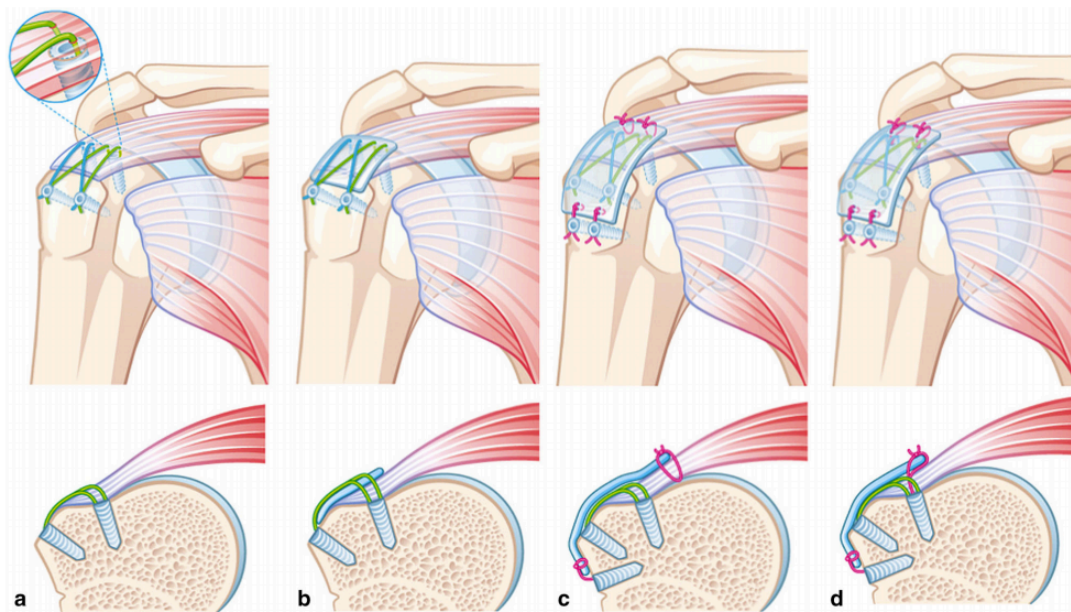


Figure 1-12. Depicting the coronal (upper) and frontal-oblique (lower) views of the different patch augmentation techniques for the study by Jung *et al.* (217).

Non-augmented control group (a), “integrated” group (b), “cover” group (c), and “hybrid” group (d).

Compared to non-augmented controls, the “cover” technique increased maximum load by 61%, the “hybrid” technique increased maximum load by

51% compared to control, and the “integrated” technique actually decreased maximum load by 28%. Both studies suggest that a technique involving the scaffold overlaying the repair is most effective at improving mechanical properties. However, a study by van der Meijden et al. (218) found no difference between the mechanical properties of a double-row augmented repair (dermal scaffold, Arthrex) compared to a non-augmented double-row repair.

1.5.1.6 Conclusion of macroscale mechanical properties

Generally, the presence of a scaffold improves the mechanical properties of a rotator cuff repair. However, this varies depending upon the type of scaffold used. Dermal scaffolds possess superior mechanical compared to SIS, but biological scaffolds have not been compared to many of the synthetic scaffolds that are commercially available. In fact, few studies of any kind have extensively compared the mechanical properties of biological and synthetic scaffolds.

Future mechanical studies should include several types of synthetic and biological scaffolds to give a more representative picture of the scaffolds that are available. Furthermore, these mechanical studies should be performed relative to native tissue, whose function the scaffolds seek to restore. This is because many previous *in vitro* studies compare the mechanical properties of scaffolds relative to each other, which is not always clinically informative. Also, care should be taken to test scaffolds in the orientation that they would be used clinically, because the properties of scaffolds can change depending upon their orientation (196).

While animal models provide a valuable option to test the mechanical properties of scaffolds, the results from such studies must be interpreted with caution. Most animal models are healthy, young quadrupeds that readily heal. In contrast, humans are bipedal and human rotator cuff tears disproportionately affect elderly patients, with chronically degenerate tissue that fails to heal.

1.5.2 Microscale mechanical properties of scaffolds

Microscale mechanical properties are properties of a material that are probed at the scale of the cell. They are important, not only for understanding the macroscale mechanical behaviour of scaffolds, but also because they can provide cues that influence biological processes.

Most cell types that reside within mammalian tissues are anchorage dependent, meaning that they need to be attached to a surface to survive. Cells attach to a surface through points of anchorage called focal adhesions, which are regions in the cell membrane where adhesion receptors concentrate (219). Focal adhesions connect to the cell's cytoskeleton and communicate information between the cell and the surrounding environment (220). As the cell attaches to the surface, it pulls upon it, generating resistance to deformation (stiffness) between the cell and the surface, which can influence a variety of cell behaviours (see below) (219). The number of focal adhesions and the way that they are distributed can also influence cell behaviours including cell spreading (221), shape (222), and differentiation (222). The

number and distribution of focal adhesions is determined by the structure and conformation of the surface to which the cells are attaching.

1.5.2.1 Young's modulus (stiffness)

The unit used to measure a material's ability to resist deformation is Young's modulus, E , which is the ratio of stress (force/area) to strain (change in length/original length). The Young's modulus of a material surface can be measured using various techniques, including atomic force microscopy (AFM).

Material stiffness has been shown to influence a variety of cell behaviours. Fibroblasts (223) and smooth muscle cells (224) spread and proliferate more on stiffer substrates. Also, many cell types migrate towards stiffer substrates, which has been observed in prostate cancer cells (225), fibroblasts (226, 227), and smooth muscle cells (228). A mathematical model correctly predicted that fibroblasts will orient themselves in an effort to maximize the effective stiffness of their environment (229) and another study demonstrated that fibroblasts will adapt the stiffness of their cytoskeleton to match that of their environment (41). Surface stiffness can also influence gene expression (230) and the differentiation of cells (231). Myofibroblasts expressed higher levels of alpha-smooth muscle actin on stiffer substrates compared to more elastic ones (230). MSCs differentiate down specific lineages depending upon surface elasticity (231), with softer matrices promoting neurogenic, stiffer matrices promoting myogenic, and rigid matrices promoting osteogenic lineage differentiation.

However, not all cell types respond favourably to stiffer substrates. Endothelial cells on collagen gels exhibited reduced network formation in stiffer gels compared to softer ones (232), and neurons form more branches on softer substrates (233). Neutrophils appear not to respond to material stiffness at all, over the orders of 3-50,000Pa (234).

1.5.2.2 Roughness (topography)

The roughness of a surface can also influence a variety of cell behaviours. Fibroblasts attach more readily to smoother surfaces than rougher ones (235, 236), and for polymer-based materials, the strength of the cell attachment is greater on rougher materials (237). Interestingly, stem cell attachment is not influenced by surface roughness (238). Even though there is a lot of evidence to suggest that fibroblasts (40, 235, 239) and osteoblasts (240) proliferate more on smoother surfaces, one study found that roughness does not influence the proliferation of fibroblasts or osteoblasts (241).

Surface roughness influences fibroblast orientation (242), morphology, but not cell volume (243). Gene expression also seems to be influenced by roughness. Fibroblasts significantly decrease the expression of migration-related genes on rougher surfaces (244) and the expression of genes in osteoblasts was most significantly changed (most genes upregulated and downregulated) on rougher surfaces (245). Similar results were seen by Leven *et al.* (246), who showed that gene expression in MSCs was most changed by rougher surfaces (genes up and down).

However, it seems like there is a threshold limit to roughness that cells can detect. Loesberg *et al.* (247) found that fibroblasts behaviour was altered by grooves deeper than 35nm, but behaviour was the same on grooves that were shallower than 35nm.

1.6 The response of tenocytes and tendon tissue to scaffolds

Scaffolds not only possess the ability to improve mechanical properties, but can also influence the behaviour of native cells following implantation. The way that tenocytes interact with an implanted scaffold likely influences the repair process. If the scaffold provides a suitable surface, native cells will begin to establish focal adhesions via trans-membrane proteins, such as integrins. Once a sufficient number of cells are present on the scaffold, they will begin to proliferate at a rate that is determined by environmental cues. Information regarding how native cells interact with scaffolds would help to determine the most appropriate clinical use of existing scaffolds and help design better scaffolds in the future.

1.6.1 The response of tenocytes to scaffolds *in vitro*

Several studies have evaluated the behaviour of tenocytes in response to commercially available scaffolds (248-252). Fini *et al.* (248) compared the response of tenocytes towards GraftJacket and tissue culture plastic (TCP) *in vitro* over 7 days. While no difference in proliferation was observed, increased expression of collagen type I, fibronectin, and TGF- β were observed in cells on GraftJacket. In a scratch-wound assay, extracts of GraftJacket reduced closure time and increased decorin expression, demonstrating that GraftJacket can influence cell behaviour both directly and indirectly. Similarly favourable results were found by another study by Fini *et al.* (250), which compared the behaviour of tenocytes derived from either healthy or glucocorticoid treated rats on GraftJacket and Restore. Over 3 days, healthy tenocytes proliferated more on GraftJacket compared to Restore and TCP, but no differences were

observed after 7 days. Interestingly, no differences in cell proliferation were seen in the glucocorticoid-derived cells between the three groups at either time point. Protein expression of type 1 procollagen enzyme synthesis, proteoglycans, and fibronectin were significantly higher in cell on GraftJacket after 7 days compared to the other two groups. The results suggest that tenocyte response is more favourable on dermal scaffolds compared to SIS.

A study by Shea *et al.* (252) also found a favourable tenocyte response towards dermal scaffolds *in vitro*. The study evaluated seven commercial scaffolds including Restore, GraftJacket, Conexa, Zimmer Collagen Repair Patch, Fascia lata, OrthADAPT and SportMesh (252). Even though tenocyte attachment was highest on Restore, these attached cells were among the least proliferative and expressed low levels of decorin, scleraxis, type I and type III collagen, in comparison to other scaffolds (252). Tenocyte attachment to Conexa was second lowest overall, but tenocyte proliferation was significantly higher on Conexa, GraftJacket, and the Zimmer Collagen Repair Patch compared to the other scaffolds. In addition, expression of *COL1A1*, *COL3A1*, *DCN*, and *SCX-B* was highest in cells on Conexa. The authors proposed that Conexa evoked the most favourable response in human tenocytes, followed by GraftJacket.

These results contrast with a study by Cook *et al.* (251), which looked at the behaviour of canine tenocytes in response to Restore, GraftJacket, CuffPatch, Permacol and TissueMend scaffolds (251). The study used subjective histological analysis and determined that more viable cells were

present on porcine SIS materials (Restore and CuffPatch) compared to the dermal scaffolds (Permacol and TissueMend). The study noted that cell migration into the scaffold was greatest on Restore and that the cells on Restore and CuffPatch were consistently associated with ECM, which was not the case in the other two scaffolds. However, the techniques used in this study were not rigorous and the subjective evaluations do not provide convincing evidence.

1.6.2 The response of tenocytes to scaffolds *in vivo*

Animal models have demonstrated that non-cross-linked dermal scaffolds can be successfully incorporated and promote the formation of organized tissue. Ide *et al.* (253) used a rat rotator cuff model to compare repair with GraftJacket against no repair (defect group). At 6 weeks, the GraftJacket group demonstrated fibroblastic ingrowth and oriented alignment with stress, compared to the defect group, which appeared disorganized and lacked fibroblastic ingrowth. At 12 weeks, the graft group collagen fibres were aligned regularly, in comparison to the defect group where collagen fibres were less aligned. Adams *et al.* (254) used GraftJacket in an inter-positional canine infraspinatus injury model. Results suggested tendon-like tissue formed at the site of implantation within 6 months. Within 6 weeks, histologic evidence suggested native cell infiltration and neotendon development. Xu *et al.* (255) used porcine dermis (Conexa) to repair the excised central third of the supraspinatus tendon in an African Green monkey model. At 6 months, the graft grossly appeared to be integrated into the host tissue and showed histologic evidence of cellular infiltration and tendon-like remodelling.

Cross-linked dermal scaffolds take more time to be incorporated into host tissue than non-cross-linked dermal scaffolds. In a sheep model, Nicholson *et al.* (256) found that after 9 weeks, cross-linked porcine dermis (Zimmer Collagen Repair Patch) was not integrated into the tissue and found a large number of fibroblasts along the surface of the scaffold. However, the scaffold was eventually fully integrated into the tissue after 24 weeks.

SIS appears to promote a somewhat inconsistent tissue response in animal studies. DeJardin *et al.* (214) used a canine infraspinatus model to study Restore and histological analysis at 3 and 6 months found tissue similar to native tendon. Similarly, Zalavras *et al.* (257) used Restore in a rat model and at 6 and 16 weeks, the Restore-augmented group demonstrated tendon-like structure with tissue ingrowth and fibroblasts oriented along the line of stress. Zheng *et al.* (258) studied Restore for rotator cuff repair in a rabbit model and at 4 weeks, histological analysis revealed that the Restore-augmented repairs were disorganized. However, by 8 weeks, 4 of 5 Restore scaffolds were totally absorbed and collagen fibres appeared as native, dense connective tissue. In contrast, Chen *et al.* (259) used a rabbit model to study Restore and after 8 weeks, histology demonstrated an abundance of disorganized granulation tissue around the repair site. Nicholson *et al.* (256) studied Restore in a sheep model and at 9 weeks, scaffolds had been completely resorbed. However, after 24 weeks, the tissue at the repair site was disorganized and contained fibroblasts, fibrocytes, chondrocytes, osteoblasts, osteocytes, and undifferentiated cells.

1.6.3 Conclusion of tenocyte and tendon tissue response to scaffolds

Studies that have evaluated the response of tenocytes to scaffolds *in vitro* and *in vivo* generally indicate that cell response is more favourable on dermal scaffolds compared to SIS. However, few studies have evaluated the response of tenocytes to synthetic scaffolds, despite many synthetic scaffolds being commercially available. Also, all of the *in vitro* and *in vivo* studies have evaluated scaffolds using healthy tenocytes and healthy tissue. Again, human rotator cuff tears are chronically degenerative and the cells within the tissue are also diseased. Future work should evaluate how diseased human cells respond to different biological and synthetic scaffolds.

1.7 Inflammatory response to scaffolds

1.7.1 In vitro studies

Several studies have looked at the response of macrophages to scaffolds *in vitro*. Grotenhuis *et al.* (260) studied how human macrophages respond to Permacol (cross-linked porcine dermis), Parietex (PET coated with porcine collagen), PET, and multifilament polypropylene. They calculated the expression of several pro-inflammatory and anti-inflammatory proteins to calculate a M1/M2 index. The authors found that Permacol and multifilament polypropylene had low M1/M2 indices, whereas Parietex and PET had higher M1/M2 indices.

It is possible that the proprietary processing methods also influence the response of macrophages towards scaffolds. Orenstein *et al.* (261) evaluated the response of human macrophages to three human dermal scaffolds (Alloderm, AlloMax, and FlexHD) and found that AlloMax and FlexHD induced a significantly increased IL-1B expression compared to Alloderm. The authors note that this could have been due to variation between donors, but is likely influenced by the processing techniques used. In a similar study, Orenstein *et al.* (262) evaluated two cross-linked porcine dermal scaffolds, (Permacol and CollaMend) and two non-cross-linked porcine dermal scaffolds, (Strattice and Surgisis). After a 7-day incubation period, the study found greater IL-1B, IL-6, IL-8, and VEGF expression on the cross-linked scaffolds compared to the non-cross-linked scaffolds. The authors suggest

that cross-linking may be responsible for inducing the pro-inflammatory response that was observed.

1.7.2 Animal studies

The way that the host's immune system responds to a scaffold has broad implications upon the success of a healed rotator cuff tendon. Following implantation, scaffolds that initiated more robust inflammatory responses have been associated with increased failure rates (254).

It seems that every type of scaffold will trigger some form of inflammatory response following implantation. However, the type and extent of this response appears to be determined by the type of scaffold implanted. Derwin *et al.* (207) used a canine infraspinatus model to evaluate an absorbable synthetic scaffold (X-Repair), and after 12 weeks, found that the implant was associated with macrophages and FBGCs, but not neutrophils or lymphocytes. Cole *et al.* (263) evaluated a non-absorbable synthetic scaffold (Biomerix RCR) in a rat model and after 6 weeks, histology demonstrated 80% tissue ingrowth and almost no inflammatory response.

Similarly mild, chronic inflammatory responses have been observed using non-cross-linked dermal scaffolds. Adams *et al.* (254) used GraftJacket in an inter-positional canine infraspinatus injury model. Within 6 weeks, chronic inflammation was observed, but was thought to be consistent with inflammation associated with surgical repair. Xu *et al.* (255) used Conexa to repair the supraspinatus tendon in an African Green monkey model and found chronic inflammation consistent with wound healing after 3 and 6

months. The study found increased vascularisation after 3 months, which decreased after 6 months, but also found a sustained elevation of alpha-gal antibodies at 4-16 times the normal level. This is thought to be caused by the alpha-gal antigens present in the porcine dermis, but did not appear to significantly influence the outcome of the repair.

Cross-linked dermal scaffolds have been associated with a more prolonged inflammatory response, but are eventually incorporated into the tissue. Nicholson *et al.* (256) used a sheep model to evaluate cross-linked porcine dermis (Zimmer Collagen Repair Patch) and after 9 weeks, found the scaffold to be associated with macrophages, FBGCs, and lymphocytes with no connective tissue inter-digitation. However, after 24 weeks, no macrophages or FBGCs were associated with the Zimmer patch, and it had been integrated into the host tissue.

The inflammatory response to porcine SIS seems to be distinct to those of synthetic and dermal scaffolds. Numerous studies have reported a robust inflammatory response towards porcine SIS scaffolds. Zheng *et al.* (258) studied the use of Restore for rotator cuff repair in a rabbit model and after 4 weeks, found the tissue to be disorganized and associated with a significantly lymphocytic infiltration. After 8 weeks, 4/5 samples had been totally resorbed, but the scaffolds that remained continued to be infiltrated by lymphocytes. Chen *et al.* (259) also used a rabbit rotator cuff model to analyse Restore, and found that scaffolds were partially resorbed after 4 weeks, and were associated with a lymphocytic infiltration, in addition to macrophages,

eosinophils, plasma and polymorphic cells. At 8 weeks, histology revealed granulation tissue around the repair site, in addition to ectopic bone formation. Nicholson *et al.* (256) used a sheep model to evaluate Restore, and found after 9 weeks, that the scaffolds had partially resorbed and FBGCs were associated with the remaining fragments. After 24 weeks, the scaffold remnants were associated with calcification and fibrocartilage formation.

The capacity for porcine SIS to promote bone and cartilage formation has been demonstrated in several studies and is particularly concerning. However, not all animal studies using SIS have been deleterious. Zalavras *et al.* (257) used a rat rotator cuff model to compare Restore-augmented repair with an unrepaired group. At 6 and 16 weeks, the Restore-augmented group demonstrated tendon-like structure and no evidence of a foreign body reaction. Dejardin *et al.* (214) used Restore in a canine model, and histological and gross analysis at 3 and 6 months showed no evidence of detachment or inflammatory response (214).

Several studies that have histologically evaluated scaffolds in animal models have noted acute inflammatory responses surrounding non-absorbable sutures (254, 256). This observation can be extended to a broader discussion on scaffolds between the balance of maintaining mechanical properties, but minimizing the chronic inflammatory response. Cross-linked biological scaffolds and non-absorbable synthetic scaffolds provide sustained mechanical support, but often at the cost of chronic inflammation. Additional studies comparing the immune response of absorbable/non-absorbable

synthetics, and cross-linked/non-cross-linked biological scaffolds would be of value to better understand this issue.

1.7.3 Conclusions of inflammatory response to scaffolds

It is clear that different scaffolds can initiate different inflammatory responses. It appears that dermal scaffolds initiate a more favourable response compared to SIS, and that non-cross-linked scaffolds are preferred to cross-linked scaffolds. It is noteworthy that we must interpret the inflammatory response from animal studies with caution. In addition to biomechanical and anatomic differences, many small animal models are inbred, genetically homogenous, and can have diminished immune systems (264). Caution must be also taken when evaluating the inflammatory response of human-derived scaffolds in non-human animals because these scaffolds would be seen as xenografts by the host animal. The evaluation of porcine scaffolds in pigs may be a more appropriate animal model to study how human allografts may behave in humans. Alternatively, human-derived macrophages could be used to evaluate the response towards synthetic and biological commercially available scaffolds. This would account for any inter-species response and would be more representative than using animal-derived cells or immortal cell lines.

1.8 Clinical studies of scaffolds

A number of clinical studies have evaluated the use of scaffolds for rotator cuff repair, but the results have varied considerably. The use of porcine SIS (specifically Restore from DePuy) has yielded mixed results. Mozes *et al.* (265) used porcine SIS in 9 patients with massive rotator cuff tears and after follow-up of 6-22 months, 8 patients had good or excellent results and only 1 patient failed repair. Similarly favourable results were reported by Metcalf *et al.* (266), who reported a healing rate of 92% (11/12) in patients who had a rotator cuff repair augmented with porcine SIS. The authors noted no complications or inflammatory responses were associated with the repairs (266).

In contrast, Malcarney *et al.* (267) initially planned to perform a prospective randomized control study using porcine SIS for rotator cuff repair, but the study had to be abandoned due to early complications. In the porcine SIS-augmented group 4 of 25 experienced an overt inflammatory reaction within two weeks of surgery (267). An erythematous wound with subcutaneous fluctuance with yellow mucinous tissue present characterized the response. Histopathological samples were composed of fibro-adipose tissue, lined by floridly, acutely inflamed granulation tissue with fibrinopurulent exudates (267). Walton *et al.* (268) published a retrospective study, using patients that were invited from the discontinued Malcarney *et al.* (267) study. After two years, patients augmented with porcine SIS experienced decreased post-repair strength, increased shoulder impingement, slower pain resolution, and no decrease in re-tear rate in comparison to patients a non-augmented control group.

Other studies have also reported an increased inflammatory response towards porcine SIS. Phipatanakul & Petersen (269) reported the outcome of 11 patients who underwent porcine SIS-augmented repair for massive rotator cuff tears. Of the 9 patients assessed at follow-up, 3 had intact repairs, 1 was partially intact and 5 repairs were not intact. Poor clinical outcomes were also reported by Sclamberg *et al.* (270), who retrospectively evaluated 11 patients who had SIS-augmented rotator cuff repairs. At 6-10 months post-operation, 10 of 11 patients suffered repair failure, with only 1 patient presenting an intact repair.

In one of the few randomized clinical trials using scaffolds for rotator cuff repair, Iannotti *et al.* (271) compared porcine SIS-augmented rotator cuff repairs with non-augmented repairs. Only 4/15 shoulders augmented with porcine SIS healed compared with 9/15 in the non-augmented control group ($p=0.11$) as measured at a minimum of 12-month follow-up (271). Also, 3 of the 15 patients who received porcine SIS experienced a sterile inflammatory reaction to the scaffold within 6 weeks of surgery (271).

Based on this string of poor clinical results, the American Academy of Orthopedic Surgeons (AAOS) recommended against using the Restore scaffold (porcine SIS scaffolds by DePuy) for the treatment of rotator cuff tears in humans (272). The Restore product line was discontinued in 2012 and stock is no longer available. Another porcine SIS scaffold, CuffPatch by Organogenesis, is also no longer commercially available.

Pericardium-derived scaffolds have also not performed well clinically. Ciampi *et al.* (273) used a bovine pericardium-derived scaffold (Tutopatch) in 49 people and after 2 years reported a 51% re-tear rate compared to 41% in the non-augmented group. After 36 months, elevation and strength was the same between the scaffold-augmented group and the control group (273).

Dermal scaffolds have performed relatively well in clinical studies, with a trend for better outcomes using human dermal scaffolds compared to porcine dermal scaffolds. Lederman *et al.* (274) used Conexa, a non-cross-linked porcine dermal scaffold, to augment rotator cuff repairs in 61 shoulders, reporting a healing rate of 66.1% (274). However, this used the criteria that a re-tear must be at least 1cm and 3 patients underwent surgical revision, with 1 patient having a deep infection. Gupta *et al.* (275) also used Conexa to repair massive or 2-tendon rotator cuff tears in 27 shoulders (26 patients). At two year follow up, 22 shoulders (21 patients) were evaluated, with 73% having intact repairs, 22% partially intact and 5% had complete re-tears (275). No cases of infection or complications were reported (275).

Several studies have also used cross-linked porcine dermis to repair rotator cuff tears, but have yielded mixed results. Badhe *et al.* (276) used the Zimmer Collagen Repair Patch and at follow-up 8/10 repairs were intact and no complications were reported (276). Similarly favourable results were reported by Cho *et al.* (277), who used Permacol to repair massive rotator cuff tears in 5 tears, with 4/5 repairs intact after an average of 20.6 months. In contrast, Soler *et al.* (278) reported a case series of 4 patients using Permacol

for rotator cuff repair, all of whom had re-tears between 3-6 months after surgery. All patients experienced an inflammatory response and worsening symptoms (278). Magnetic resonance imaging (MRI) scans revealed the recurrent tears, inflammatory changes, and significant fluid pooling in the sub deltoid bursa area of all patients (278). One of the patients required draining by ultra-sound guided aspiration, but the aspirate was negative for infection (278). Two of the patients underwent revision surgery to remove the implant, and histologic analysis revealed fibrin-like material on a background of chronic inflammation (278).

Human dermal scaffolds have performed well in clinical studies. Barber *et al.* (279) reported a prospective randomized controlled study of GraftJacket (human dermis) for rotator cuff repair. At a mean follow-up of 24 months, the study demonstrated intact repairs in 85% of the augmented group compared to 40% in the non-augmented group (279). Bond *et al.* (280) reported a retrospective study on 16 patients with massive, contracted, immobile rotator cuff tears treated with GraftJacket augmentation. After 12 months, 13/16 patients had intact repairs (280). Of the 3 that failed, none had to have the graft removed and all were satisfied with the procedure because it had decreased pain levels (280). A biopsy specimen was taken from one of the patients who failed to repair after she fell, and the biopsy revealed neotendon formation (280). Burkhead and colleagues (281) also reported favourable results using GraftJacket in 17 patients with either primary massive rotator cuff tear (11 patients) or recurrent massive rotator cuff tears (6 patients). Of the 12 patients who were evaluated using advanced imaging studies, only 3

presented with a re-tear (281). Flury *et al.* (282) used GraftJacket MaxForce Extreme to repair RC tears in 8 shoulders. At 6 months, 5/8 were healed, 1/8 were completely re-ruptured and 2/8 had incomplete re-ruptures, with no complications reported (282).

Human dermal scaffolds other than GraftJacket have also proved to be effective in clinical studies. Petri *et al.* (283) used a different human acellular dermal matrix (Arthroflex) to augment tendon repairs and 5/6 patients had intact repairs after an average of 9.9 months. Yoon *et al.* (284) compared group 1 (21 patients who had bone marrow stimulation with patch augmentation using a human dermal scaffold (AlloCover, Hans Med)) with group 2 (non-augmented conventional rotator cuff repair). At follow-up, re-tear rate was significantly lower in group 1 (4/21) compared to group 2 (25/54) (284).

Fascia lata has also appeared to be an effective biological scaffold for rotator cuff repair. Agrawal (285) published a retrospective case series using an acellular human graft (AlloPatch HD Hydrated Ultra Thick, MTF Sports Medicine) and after an average of 16.8 months post-operation, 12/14 patients had intact repairs.

Synthetic scaffolds have also proven to be effective in reducing re-tear rates. Petriccioli *et al.* (286) used Sportmesh (also known as Artelon) to repair subscapularis tears in 10 patients with 9/10 patients showing intact repairs at follow-up. Marberry (287) used Artelon Tissue Reinforcement in 17 patients with challenging chronic rotator cuff tears. At 1-year follow-up, scans revealed repair failure in 3 patients, one of whom had a stroke and fell on

their shoulder. The 13 patients with intact repairs at 1 year after surgery, also had intact repairs 2 years after surgery (287).

X-Repair has shown mixed results clinically. Proctor *et al.* (288) used X-Repair to repair large-massive rotator cuff tears arthroscopically in 18 patients. After 12 months, 15/18 had intact repairs, and, after 42 months 14/18 had intact repairs (288). In contrast, Lenart *et al.* (289) treated massive rotator cuff tears in 16 patients with X-Repair. After an average of 1.5 years, the re-tear only 38%(5 patients) had intact repairs.

Other various synthetic scaffolds have proven to be relatively effective. Audenaert *et al.* (290) prospectively operated on 41 patients with massive cuff tears (greater than 4cm), treated in a primary operation (no previous operations) with non-absorbable patch (Mersilene mesh, Ethicon, which was folded into 8-layer mesh and cut to size of defect) transosseously with SAD. After a mean duration of 43 months, only 3/41 had a recurrent tear (290).

Encalada-Diaz *et al.* (291) published a report of 10 patients with cuff tears undergoing repairs with a Biomerix patch (novel non-absorbable reticulated polycarbonate polyurethane patch). MRI at 12 months showed healing in 90%; only one patient had re-tear. No adverse events associated with patch, including absence of fibrosis, mechanical symptoms or visible subacromial adhesions.

Nada *et al.* (292) repaired massive tears in 21 patients with an augmented polyester ligament (Dacron). Mean follow up was 36 months, there were 2 failures—1 due to ruptured ligament and 1 to deep-seated

infection. MRI scans performed at follow-up confirmed intact and thickened bands in 15/17 patients.

Hirook *et al.* (293) used Gore-tex to repair 28 shoulders in 27 patients. At an average follow-up of 44 months, only 3 re-tears were reported.

1.8.1 Conclusions of clinical studies

Porcine SIS seems to promote an unfavourable inflammatory response in humans, which can lead to poor clinical outcomes. However, dermal and synthetic scaffolds generally reduced rotator cuff re-tear rates. The current limitation in many clinical studies is that they do not have a non-augmented control arm to definitely demonstrate efficacy. Future clinical studies should have a non-augmented control arm and would ideally compare the performance of several different commercially available scaffolds.

1.9 Aims and hypotheses of this thesis

This thesis aims to characterise macroscopic mechanical properties (tensile strength, suture retention strength, and storage modulus (in shear)) and microscopic mechanical properties (Young's modulus, loss tangent, and roughness) of a subset of commercially available scaffolds. Furthermore, this thesis aims to understand how human tenocytes and macrophages respond to these scaffolds *in vitro*. This thesis hypothesizes the following:

1. Synthetic scaffolds would have equivalent macroscopic mechanical properties to supraspinatus tendon. Biological scaffolds would have equivalent microscopic mechanical properties to supraspinatus tendon.
2. More tenocytes will attach and proliferate on biological scaffolds than synthetic scaffolds. Biological scaffolds will promote tenocytes to express higher levels of COL1A1 and COL3A1 mRNA than synthetic scaffolds. Biological scaffolds will promote an elongated and natural tenocyte morphology.
3. Synthetic scaffolds would cause human macrophages to express the highest levels of pro-inflammatory mediators at the mRNA and protein levels. Pro-inflammatory mediators released by macrophages on synthetic scaffolds would downregulate tendon ECM genes in patient-derived tenocytes. More foreign body giant cell would form on synthetic scaffolds compared to every other scaffold type.

Chapter 2 : The mechanical properties of rotator cuff scaffolds

2.1 Abstract

2.1.1 Background

Re-tearing following rotator cuff surgery is a major clinical problem. Numerous scaffolds are being used to try and reduce re-tear rates. However, few have demonstrated clinical efficacy. We hypothesise that this lack of efficacy is partly due to mechanical properties that are inadequate to support a rotator cuff repair. We therefore compared the macro and micro mechanical properties of seven commercially available scaffolds to those of healthy human supraspinatus tendons, whose function they seek to restore.

2.1.2 Materials and methods

The clinically approved scaffolds tested were X-Repair, LARS ligament, Poly-Tape, BioFiber, GraftJacket, Permacol, and Conexa. Fresh-frozen cadaveric human supraspinatus tendon samples were used as a comparator. Macro mechanical properties were determined using tensile testing, suture pull out, and rheometry. Results from these were also used to determine macroscale, Young's modulus and loss tangent. Scanning probe microscopy and scanning electron microscopy were performed to assess mechanical properties at the microscale including morphology, Young's modulus, loss tangent, and roughness.

2.1.3 Results

None of the scaffolds tested adequately approximated both the macro and micro mechanical properties of healthy human supraspinatus tendon. Macroscale mechanical properties were insufficient to restore load-bearing function. The best performing scaffolds on the macroscale (X-Repair and LARS ligament) had poor microscale properties. Scaffolds approximating tendon properties on the microscale (BioFiber and biological scaffolds) had poor macroscale properties.

2.1.4 Conclusions

Our results suggest that existing commercial scaffolds require modification to restore mechanical function at both the micro and macro scales. We suggest that combining the macro mechanical properties of a synthetic scaffold with the micro mechanical properties of a biological scaffold would better approximate native tendon tissue. However, this could not be achieved using existing scaffolds in their current form. Future work should focus on creating new scaffolds with desirable mechanical properties across all hierarchical levels, which meet the demands of the target tissue. It also highlights the need for scaffolds that are specifically designed to meet the demands of the target tissue.

2.2 Introduction

Even though scaffolds have been commercially available for the past three decades, few have demonstrated convincing clinical efficacy for rotator cuff tendon repair. While some clinical studies have yielded promising results (266, 275, 287, 294), only two prospective randomised control trials have been conducted (295, 296); one demonstrated slight clinical improvement with scaffold augmentation (295), but the other reported worse outcomes with scaffold use (296). Many scaffolds were not specifically designed for the loading environment of the rotator cuff and the lack of reported efficacy might be explained by the inadequate mechanical properties of commercial scaffolds.

Macroscale mechanical properties of scaffolds are bulk properties that include tensile strength, suture retention, and shear modulus. Macroscale properties are influenced, not only by the material, but also by the form, orientation, and structure of the scaffold. Macroscale properties of scaffolds are important to determine because they reflect the scaffold's capacity to cope with the types of forces it would experience following implantation *in vivo*. Of these tests, suture pull-out is particularly important. This is because the scaffold-suture interface is prone to stress concentration, making suture pull-out the most common form of rotator cuff surgical failure (200).

Microscale mechanical properties such as Young's modulus, roughness, and loss tangent are properties evaluated at the micron scale and can influence cell behaviour (234, 297-299). While the precise mechanisms

underlying these behaviours continue to be investigated, it has been shown that cells interpret surface properties through cell adhesion complexes, which trigger changes in cell behaviour (300, 301). For example, fibroblasts attach more readily to smoother surfaces (235, 236), but the strength of the attachment is greater on rougher surface (237). In addition, cells can detect the stiffness (Young's modulus) of the surface they are on and alter their behaviour accordingly. Fibroblasts proliferate more on stiffer substrates (223) and preferentially migrate towards surfaces of higher stiffness (226, 227). Despite the evidence that microscale material properties can influence the behaviour of cells, these properties have not been well studied in the context of rotator cuff scaffolds.

A number of previous studies have investigated the macroscale mechanical properties of scaffolds (178, 195, 199, 201, 302). Barber *et al.* (201) compared the suture pull-out strengths of five biological scaffolds and found that dermal scaffolds had better suture retention than SIS scaffolds. Derwin *et al.* (195) also reported that dermal scaffolds are thicker and stiffer than SIS scaffolds, while Chaudhury *et al.* (199) demonstrated that dermal scaffolds were also stronger than SIS and synthetic scaffolds. Barber *et al.* (302) also tested seven scaffolds to failure in tensile testing and suture pull-out, reporting that dermal and fascia lata scaffolds had superior mechanical properties compared to pericardium and synthetic scaffolds. Implanted scaffolds are exposed to degradative enzymes and a study by Annor *et al.* (178) tested the tensile properties of nine biological scaffolds following enzymatic digestion for different lengths of time. The study demonstrated

that cross-linked scaffolds (dermal and pericardium) maintained their mechanical properties much better than the non-cross-linked equivalents (178).

Even though more than 20 scaffolds are available to augment rotator cuff repair, previous mechanical studies have focused on a small group of biological scaffolds and have largely overlooked synthetic scaffolds. Furthermore, no study has directly compared the macroscale mechanical properties of scaffolds to those of human supraspinatus tendon, whose function they seek to restore. While it is clear that microscale mechanical properties can influence mechanical performance and cell response, few previous studies have characterised the microscale mechanical properties of scaffolds or compared them to native tissue.

2.3 Aims and hypotheses

The purpose of this study was to compare the macroscale and microscale mechanical properties of seven commercially available rotator cuff scaffolds and healthy human supraspinatus tendons.

It was hypothesised that the macroscale properties of synthetic scaffolds would be equivalent to those of supraspinatus tendon and the microscale properties of biological scaffolds would be equivalent to those of supraspinatus tendon.

2.4 Materials and methods

2.4.1 Material selection and preparation

Seven scaffolds were selected as a representative sample of scaffolds in current clinical use for rotator cuff repair (Table 2-1). They included four synthetic scaffolds (X-Repair, LARS ligament, Poly-Tape, and BioFiber) and three biological scaffolds (GraftJacket, Permacol, and Conexa). All scaffolds were prepared according to the manufacturers' instructions. Under sterile conditions, samples were then soaked in phosphate-buffered saline (PBS, Sigma-Aldrich, USA) for 5 minutes and briefly dabbed dry with a paper towel prior to testing. This was performed to ensure uniform scaffold preparation.

Supraspinatus tendons were collected from five fresh-frozen human cadaveric specimens aged 60-93 years. Ethical approval was received from Cambridgeshire and Hertfordshire Health Research Authority (Ethics NO. 15/EE/0304). Tendon samples included in the study had no macroscopic signs of tissue damage.

2.4.2 Tensile testing

Dumbbell-shaped scaffolds are frequently used in tensile mechanical testing because they give provide a predictable point of material failure and help to reduce stress concentration at the grips (199, 303, 304). A preliminary investigation using dumbbell-shaped pieces of scaffolds yielded inconsistent results between different pieces of the same scaffold. This was likely due to the difficulty associated with creating dumbbell-shaped pieces of similar size and shape from the limited quantities of scaffold available. Rectangular-

shaped pieces of scaffolds have also been used previously (195, 302), and as, in preliminary testing, they generated more reproducible data, were used for this study. Pieces of each material (n=5) measuring 2cm x 0.5cm were cut and oriented in the direction they would be used clinically.

A 660N tensile stage (Deben, UK) was used to test the samples to failure. Grip-to-grip separation at the beginning of each test was 1cm. The stage extended at a rate of 0.5mm/min and samples were tested to failure. Load at failure was recorded. Cross-sectional area measurements derived from scanning electron microscopy (SEM) images were used to calculate the maximum Young's modulus (equation below).

$$E = \left(\frac{F}{A_0}\right) / \left(\frac{\Delta L}{L_0}\right)$$

Equation 2-1. Equation for Young's modulus (305).

E=Young's modulus (N/m²)

F=Maximum force under tension (N)

A₀=Original cross-sectional surface area (m²)

ΔL= Change in length (m)

L₀=Original length (m)

2.4.3 Suture pullout testing

Materials were cut to 2cm x 0.5 cm (n=5) in the orientation that they would be used clinically. A single number 2 polydioxanone suture (PDS II suture, Ethicon, USA) was passed through the centre of each scaffold, 2.5mm from the distal end. A 10kN Zwick tensile testing machine (Zwick Roell Group, USA) was used to test each sample. Approximately 5mm of the proximal

portion of each material was placed into the lower stationary grip and the suture was left untied and clamped in the upper grip. The grip-to-grip separation was maintained at approximately 1.5cm. The sutures and material samples did not move or slip once they were within the grips. The upper grip moved at 2mm/min until the suture pulled through the material or the suture snapped. The force at which the suture pulled out or snapped was recorded.

2.4.4 Rheometry testing

Rotator cuff tendons can experience significant shear forces (306). Therefore, scaffolds used to repair rotator cuff tendons can also experience such shear forces. To determine the propensity of each scaffold to deform in response to an applied shear stress, the shear modulus for each scaffold was determined using a rheometer. Storage modulus (the propensity of a material to store energy) and loss modulus (the propensity of a material to lose energy) were also determined during rheometry (Appendix A). Dividing the loss modulus by the storage modulus gives the loss tangent ($\text{loss tangent} = \text{loss modulus} / \text{storage modulus}$). The loss tangent provides a quantitative ratio of energy stored to energy dissipated by each scaffold.

For this study, an Anton Paar Physica MCR 301 rheometer (Anton Paar, Austria) was used. Five 8mm circles were cut from each material using a biopsy punch. Samples were kept at 37°C and subjected to a constant normal force of 0.2N. The upper plate moved sinusoidally at angular frequencies 0.5-100 rad/s.

2.4.5 Scanning electron microscopy (SEM)

Two samples measuring 0.5cm by 0.5cm of each scaffold and supraspinatus tendon were prepared for SEM. Samples were fixed in 2.5% glutaraldehyde (Sigma-Aldrich, USA) for 10 minutes. Samples were rinsed twice in deionised water before being dehydrated through a graded ethanol (Sigma-Aldrich, USA) series for 2 minutes at each concentration (40%, 70%, 90%, 95%, 100%). Next, 100 μ L of hexamethyldisilazane (Alfa Aesar, USA) was added to each sample and left for 24 hours. Samples were coated in gold using a SC7620 Mini Sputter Coater System (Quorum Technologies Ltd, UK). Images were captured at 50X and 1000X magnifications using an Evo LS15 Variable Pressure Scanning Electron Microscope (Carl Zeiss AG, Germany). Material thicknesses were determined from cross-sectional images using ImageJ (National Institute of Health, USA).

2.4.6 Scanning probe microscopy (SPM)

A 0.5cm by 0.5cm piece of each material was cut under sterile conditions. Each piece was attached to a glass slide (Sigma-Aldrich, USA) using 100 μ L of epoxy resin (Evo-stick, UK).

Scanning probe microscopy was performed on a MFP-3D Atomic Force Microscope (Asylum Research, High Wycombe, UK), with an AC160TS probe ($k=63\text{kN}$, Olympus, Japan). Measurements were performed using AMFM nanomechanical mapping (307) and loss-tangent imaging (308), utilising the shift in the probe's resonant frequencies dependent on the strength of the interaction, or tip-sample contact force (309). A tip correction factor was

calculated, based on the known modulus of polydioxanone (2GPa, Ethicon, USA), assuming consistent punch geometry. The tip correction factor produced a calculated punch radius of 24nm. Five $10\mu\text{m} \times 10\mu\text{m}$ (512x512 pixels) areas were then randomly selected and measured from each material. Surface values for Young's modulus, loss tangent, and roughness were recorded for each image.

The average roughness (R_q) was determined from the average deviations in height of the probe from the base level (zero). Two areas were used for roughness measurements. The total roughness measured entire images ($10\mu\text{m} \times 10\mu\text{m}$), which included the deviation in height due caused by the curvature of the fibres in synthetic scaffolds. The central roughness measured only the central strip of each image ($2\mu\text{m} \times 10\mu\text{m}$) to give a measurement of the roughness on the surface of each fibre of the synthetic scaffolds without the effect of fibre curvature.

2.4.7 Statistical analysis

For each experimental test, normality was determined using a Kolmogorov-Smirnov test. For each experiment, the data from each material tested appeared normally distributed. A one-way ANOVA was performed, followed by a Tukey post-hoc test to compare all materials with each other. Statistical significance was determined at $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ and 95% confidence intervals (CIs) of minimum difference were reported. GraphPad PRISM was used for all statistical analyses. Statistics in figures are represented by small and capital letters. A capital letter (e.g. "A") designates

that material as significantly greater than every other material with the corresponding small letter (e.g. "a").

2.5 Results

Table 2-1. Characteristics of biological and synthetic scaffolds used to augment rotator cuff repairs. *Represents approximate measurements.

Name	Manufacturer	Material	Uncut Length x Width (mm)	Thickness (mm)
X-Repair	Synthasome	Poly-l-lactic acid	43 x 12	1.39 +/-0.21
LARS Ligament	LARS	Polyethylene terephthalate	170* x 30*	0.80 +/-0.13
Poly-Tape	Neoligaments	Polyethylene terephthalate	500 x 40	0.47 +/-0.15
BioFiber	Tornier	Poly-4-hydroxybutyrate	50 x 25	1.11 +/-0.19
GraftJacket	Wright Medical	Human dermis	70 x 40	1.56 +/-0.14
Permacol	Covidien	Porcine dermis	50 x 50	1.20 +/-0.16
Conexa	Tornier	Porcine dermis	50 x 50	1.36 +/-0.08
Human supraspinatus tendon	-	Human tendon	-	2.00 +/-0.33

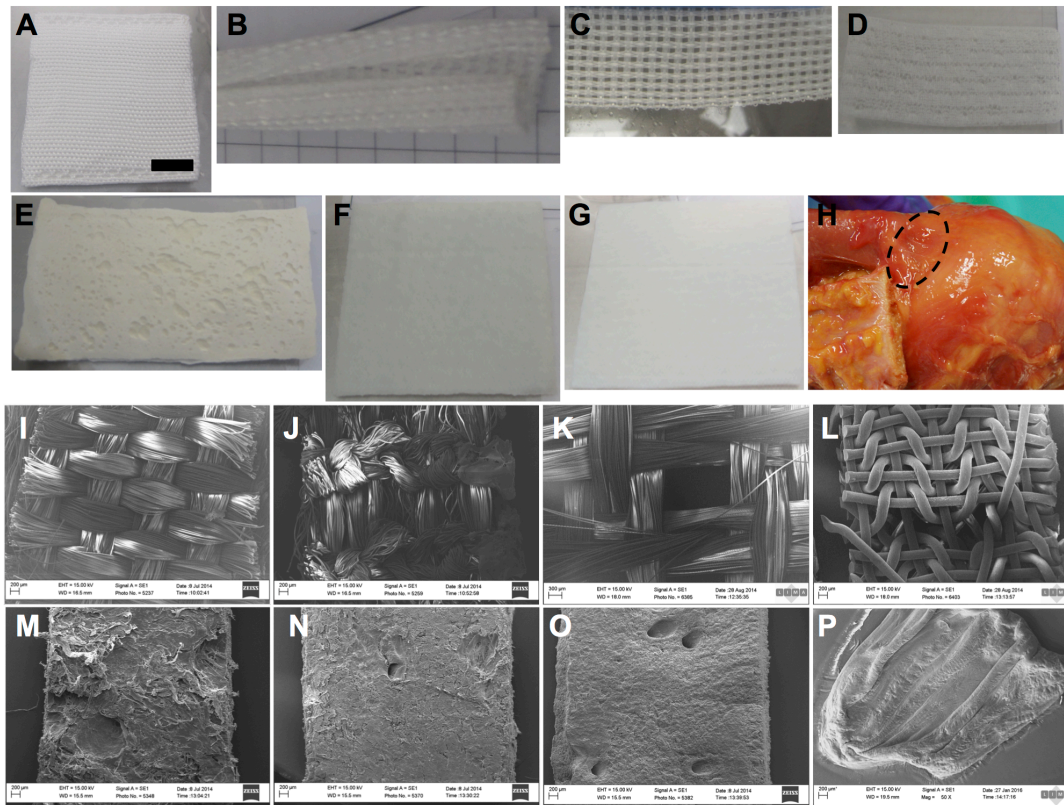


Figure 2-1. Macroscopic images of materials tested.

Macroscopic pictures of X-Repair (A), LARS Ligament* (B), Poly-Tape* (C), BioFiber (D), GraftJacket (E), Permacol (F), Conexa (G), human humeral head with intact supraspinatus tendon denoted by dashed line (H). Scale bar for macroscopic pictures (A-H) is 10mm. *Images show only part of the scaffold. SEM micrographs of materials tested: X-Repair (I), LARS Ligament (J), Poly-Tape (K), BioFiber (L), GraftJacket (M), Permacol (N), Conexa (O), human supraspinatus tendon (P). Magnification=50X and scale bars are 200µm (scale bar for (K) is 300µm).

2.5.1 Tensile testing

Representative stress-strain curves of all materials tested are presented in figure 2-2A. Compared to supraspinatus tendon, the synthetic scaffolds of X-Repair, LARS ligament, and Poly-Tape reached their maximum stress at lower strains. In contrast, BioFiber and the biological scaffolds GraftJacket, Permacol, and Conexa reached their maximum stress at higher strains.

For load to failure tests, the synthetic scaffolds X-Repair ($p \leq 0.001$, 95% CI: 42, 152) LARS ligament ($p \leq 0.01$, 95% CI: 25, 135) and Poly-Tape ($p \leq 0.05$, 95% CI: 11, 121) failed at forces that were significantly higher (i.e. at least two-fold increase) than all other scaffolds (figure 2-2B). Figure 2-2B represents a boxplot with minimum and maximum values at the extremes, with the lower quartile, median, and upper quartile represented by the boxes. Interestingly, BioFiber, the other synthetic scaffold tested, failed at forces significantly lower than the other three synthetic scaffolds tested (figure 2-2B). It is unclear precisely why this is, but differences in polymer and scaffold construction may help explain this. However, none of the scaffolds tested failed at forces close to that of human supraspinatus tendon, which failed at an average of 312N, which is 1.6 times the force of the strongest scaffold ($p \leq 0.001$, 95% CI: 62, 172).

The Young's modulus for LARS ligament ($p \leq 0.01$, 95% CI: 87, 508) and Poly-Tape ($p \leq 0.05$, 95% CI: 17, 438) were significantly higher than the other materials tested (figure 2-4A). LARS ligament demonstrated a Young's

modulus that was significantly higher than that of Poly-Tape (1.7 fold higher, $p \leq 0.01$, 95% CI: 87, 508).

2.5.2 Suture pullout testing

The maximum force at failure for suture pull-out was greater for X-Repair ($p \leq 0.001$, 95% CI: 12, 54) than any other scaffold tested, except LARS ligament (figure 2-2C). However, none of the scaffolds were able to match the forces of suture pull out for supraspinatus tendon ($p \leq 0.001$, 95% CI: 44, 86) (figure 2-2C). It must be noted that while sutures pulled through all the scaffolds, failure of tendon samples was due to sutures snapping rather than pulling through the tendon tissue. Figure 2-2C represents a boxplot.

In graphs throughout this chapter, synthetic scaffolds are represented in blue, biological scaffolds are represented in red, and supraspinatus tendons are represented in black.

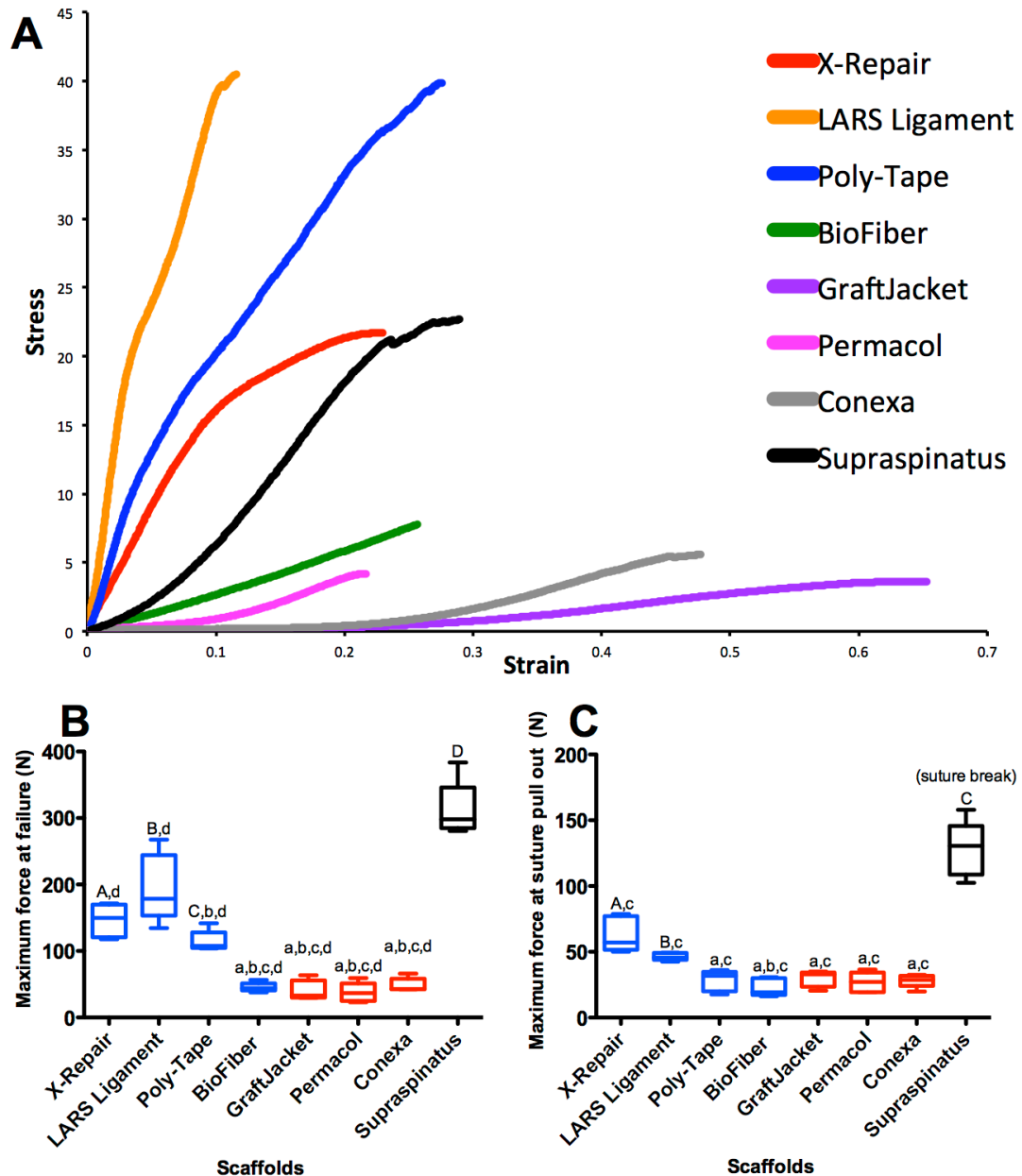


Figure 2-2. Macroscopic mechanical testing of scaffolds.

Representative stress-strain curves of materials derived from tensile mechanical testing (A). Force at failure of materials tested represented by a boxplot (B) where $Aa \leq 0.001$, $Bb \leq 0.01$, $Cc \leq 0.05$, $Dd \leq 0.001$. Maximum force at suture pull out or suture snap represented by a boxplot (C) where $Aa \leq 0.001$, $Bb \leq 0.05$, $Cc \leq 0.001$. In (C), please note that sutures pulled through all scaffolds tested, but did not pull through the supraspinatus tendon and snapped instead. Blue denotes synthetic scaffolds, red denotes biological scaffolds, and black denotes human supraspinatus tendons.

2.5.3 Rheometry testing

The storage modulus and loss modulus were determined using rheometry. By dividing the loss modulus by the storage modulus, the loss tangent (in shear) was calculated. The loss tangent of Poly-Tape was significantly higher than all scaffolds ($p \leq 0.001$, 95% CI: 0.034, 0.14) except GraftJacket (figure 2-5A). GraftJacket has a loss tangent greater than LARS ligament, BioFiber, and X-Repair ($p \leq 0.001$, 95% CI: 0.032, 0.14) (figure 2-5A). Supraspinatus tendon had a loss tangent greater than all scaffolds tested ($p \leq 0.05$, 95% CI: 0.00089, 0.10). Figure 2-5A represents a boxplot.

2.5.4 Scanning electron microscopy (SEM)

Scanning electron micrographs revealed that the fibres of X-Repair (figure 2-3A), LARS ligament (figure 2-3B), and Poly-Tape (figure 2-3C) are smooth and featureless. The surface of BioFiber fibres (figure 2-3D) appeared rougher, with more features at the microscale. The biological scaffolds GraftJacket (figure 2-3E), Permacol (figure 2-3F), and Conexa (figure 2-3G) had heterogeneous surfaces that appeared to be composed of fibrous proteins. The surface of the supraspinatus tendon (figure 2-3H) showed characteristic bundles of parallel and aligned collagen fibrils.

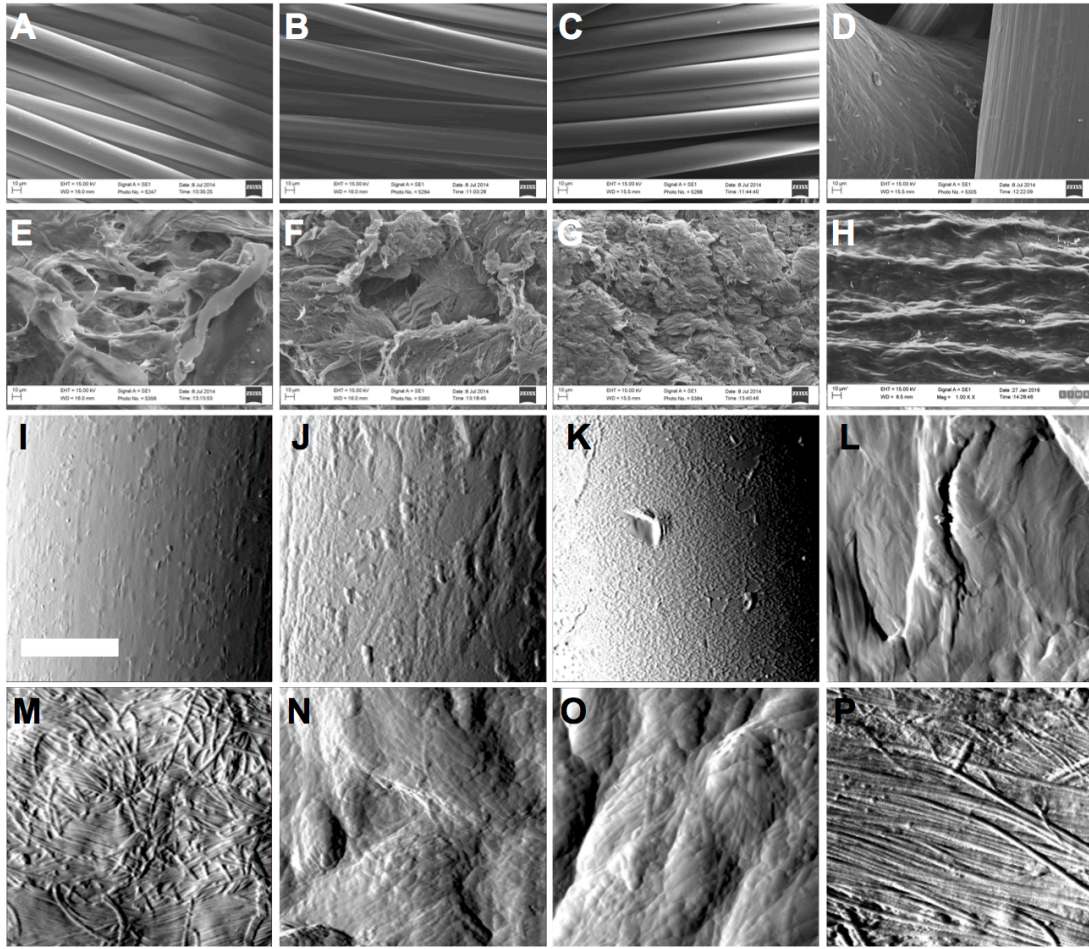


Figure 2-3. Scanning electron microscopy (A-H) and scanning probe microscopy images (I-P) of scaffolds.

Scanning electron microscopy images: X-Repair (A), LARS Ligament (B), Poly-Tape (C), BioFiber (D), GraftJacket (E), Permacol (F), Conexa (G), human supraspinatus tendon (H). Images are 1000X magnification and scale bar=10µm. **Scanning probe microscopy images:** X-Repair (I), LARS Ligament (J), Poly-Tape (K), BioFiber (L), GraftJacket (M), Permacol (N), Conexa (O), human supraspinatus tendon (P). Images are of the amplitude retrace. Images are 10µm x 10µm. Scale bar= 4µm.

2.5.5 Scanning probe microscopy (SPM)

SPM images revealed marked differences in the morphology of scaffold surfaces. The surface features on the synthetic fibres of X-Repair (figure 2-3I) and Poly-Tape (figure 2-3K) appeared to be slightly less pronounced compared to LARS ligament (figure 2-3J) and BioFiber (figure 2-3L). The

surfaces of Permacol (figure 2-3N) and Conexa (figure 2-3O) were similar with large bundles of protein, but few discernable collagen fibres. In contrast, collagen fibres were identifiable on the surface of GraftJacket (figure 2-3M) and human supraspinatus (figure 2-3P), but were more aligned in the supraspinatus than in GraftJacket.

The microscale Young's modulus of X-Repair ($p \leq 0.01$, 95% CI: 0.20, 1.07) was significantly greater than all other scaffolds tested (figure 2-4B). The localised Young's moduli of LARS ligament ($p \leq 0.001$, 95% CI: 0.30, 1.17) and Poly-Tape ($p \leq 0.01$, 95% CI: 0.17, 1.03) were greater than those of BioFiber, GraftJacket, Permacol, and supraspinatus tendon (figure 2-4B). The local Young's modulus of Conexa ($p \leq 0.05$, 95% CI: 0.018, 0.88) was significantly greater than BioFiber, GraftJacket, and Permacol (figure 2-4B). Figure 2-4B is a boxplot.

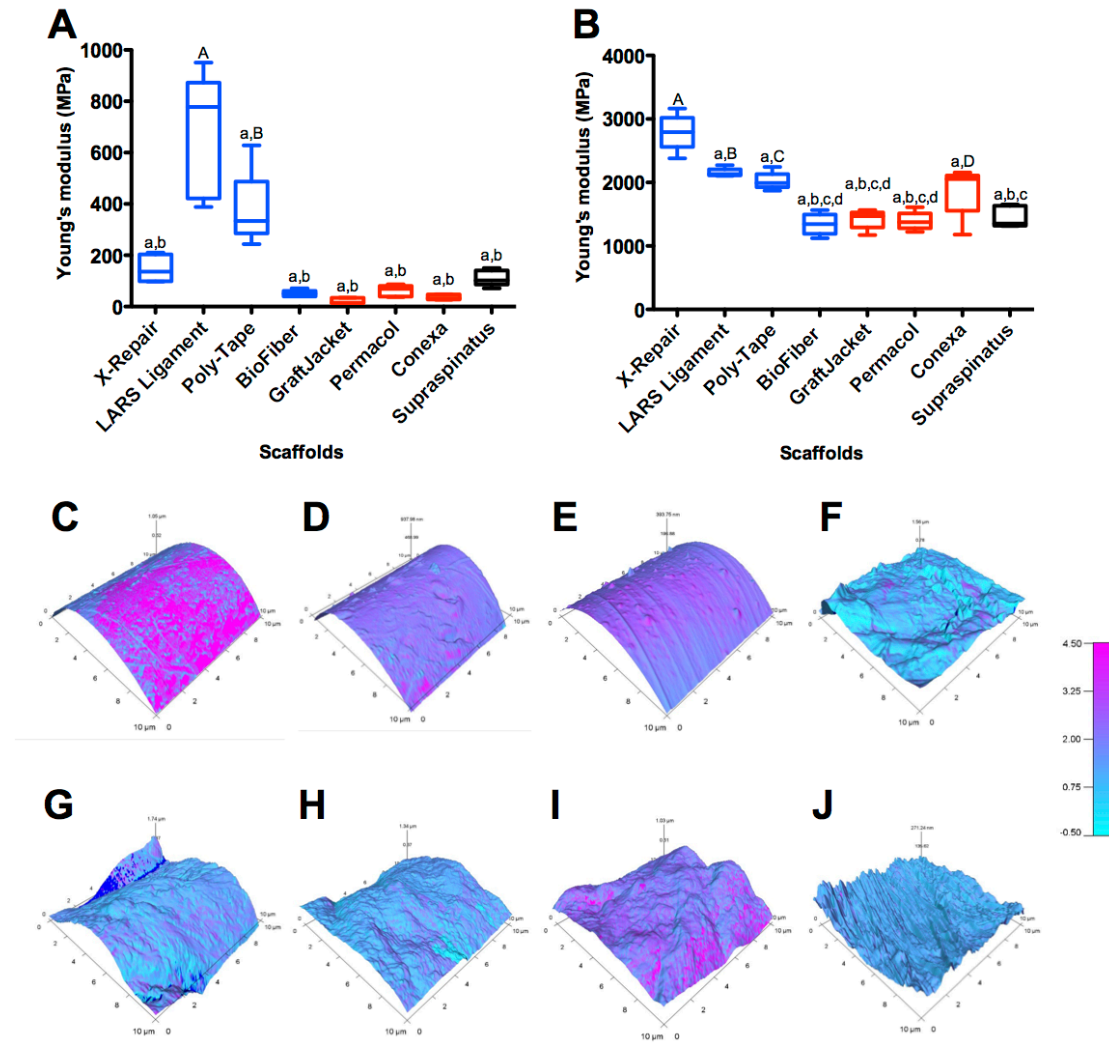


Figure 2-4. Macroscopic and microscopic Young's moduli.

Young's modulus derived from macroscopic mechanical testing represented by a boxplot (A) where $Aa \leq 0.01$, $Bb \leq 0.05$. Young's modulus derived from Scanning probe microscopy represented by a boxplot (B) where $Aa \leq 0.01$, $Bb \leq 0.001$, $Cc \leq 0.01$, $Dd \leq 0.05$. Blue denotes synthetic scaffolds, red denotes biological scaffolds, and black denotes human supraspinatus tendons. Scanning probe microscopy images mapping the Young's modulus of the surface of materials: X-Repair (C), LARS Ligament (D), Poly-Tape (E), BioFiber (F), GraftJacket (G), Permacol (H), Conexa (I), human supraspinatus tendon (J). Magenta-cyan scale bar presented on right of figure.

The localised loss tangent of X-Repair ($p \leq 0.001$, 95% CI: 132, 512) was significantly higher than all other materials tested (figure 2-5B). Figure 2-5B is a boxplot.

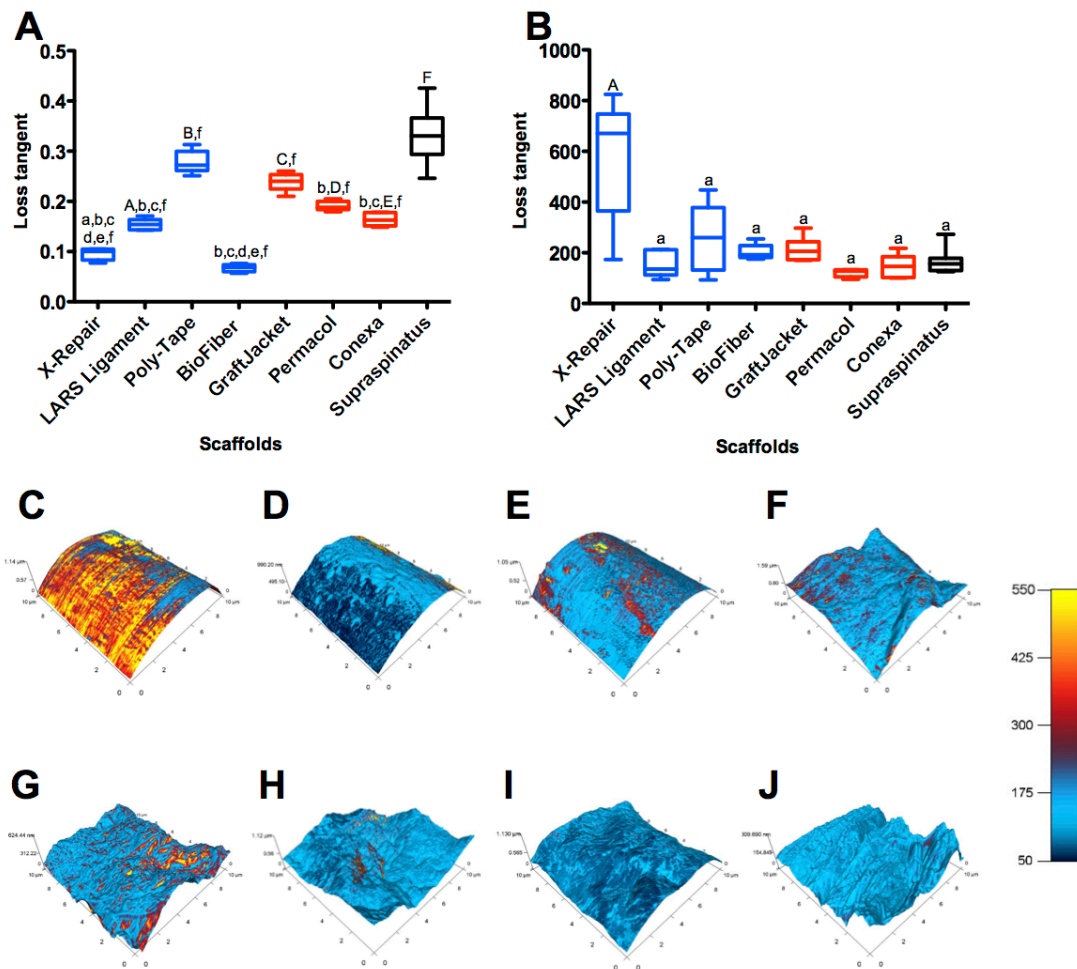


Figure 2-5. Macroscopic and microscopic loss tangents.

Loss tangent derived from macroscopic mechanical testing represented by a boxplot (A) where Aa $\leq$ 0.05, Bb $\leq$ 0.001, Cc $\leq$ 0.001, Dd $\leq$ 0.001, Ee $\leq$ 0.01, Ff $\leq$ 0.05. Loss tangent derived from scanning probe microscopy represented by a boxplot (B) where Aa $\leq$ 0.001. Blue denotes synthetic scaffolds, red denotes biological scaffolds, and black denotes human supraspinatus tendons. Scanning probe microscopy images mapping the loss tangent of the surface of materials: X-Repair (C), LARS Ligament (D), Poly-Tape (E), BioFiber (F), GraftJacket (G), Permacol (H), Conexa (I), human supraspinatus tendon (J). Yellow-blue heat scale bar presented on right of figure.

Total roughness (figure 2-6A) showed that BioFiber had the lowest surface roughness. However, after accounting for fibre curvature, the roughness of biological scaffolds was significantly higher than synthetic scaffolds (figure 2-6B) ($p \leq 0.001$, 95% CI: 27, 78). Figures 2-6A and 2-6B are boxplots.

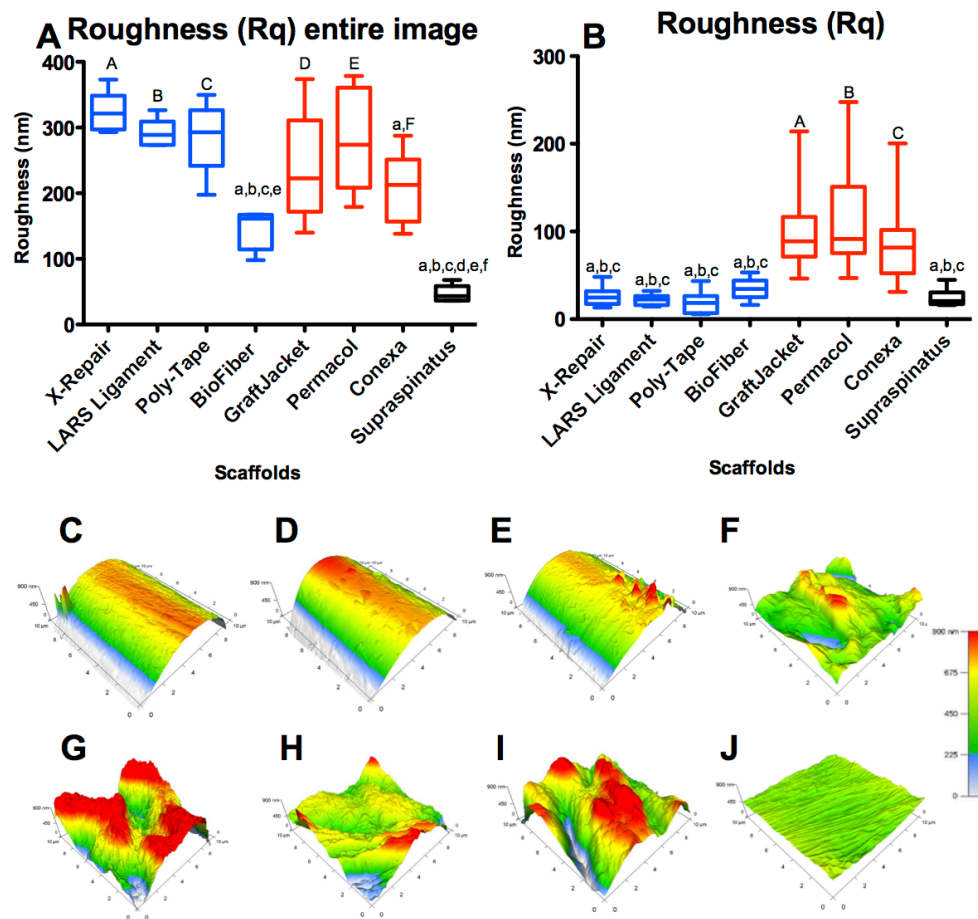


Figure 2-6. Microscale surface roughness of scaffolds.

Average roughness (Rq) was determined using scanning probe microscopy images. Roughness was determined on entire image represented by a boxplot (A) where Aa $p \leq 0.05$, Bb $p \leq 0.01$, Cc $p \leq 0.01$, Dd $p \leq 0.001$, Ee $p \leq 0.01$, Ff $p \leq 0.01$. Roughness was also determined for the central portion (2µm by 10µm) of each image represented by a boxplot (B) where Aa ≤ 0.01 , Bb ≤ 0.01 , Cc ≤ 0.01 . Blue denotes synthetic scaffolds, red denotes biological scaffolds, and black denotes human supraspinatus tendons. Scanning probe microscopy images mapping the roughness (Rq) of the surface of materials: X-Repair (C), LARS Ligament (D), Poly-Tape (E), BioFiber (F), GraftJacket (G), Permacol (H), Conexa (I), human supraspinatus tendon (J). Red-yellow-green-blue scale bar presented on right of figure.

2.6 Discussion

By comparing seven commercially available scaffolds clinically utilised for rotator cuff repair with healthy human supraspinatus tendon, we found significant differences at both the macroscale and microscale. While synthetic scaffolds better approximated the macroscale mechanical properties of supraspinatus tendon and biological scaffolds approximated the microscale mechanical properties, no scaffold fully approximated the properties of native tissue.

‘Bridging’ refers to the process of using a scaffold to replace missing tendon tissue to connect muscle to bone. Although scaffolds are not licensed for use as bridging devices (310), they have been used for this purpose in rotator cuff repair (278, 311, 312). Even when used as an augmentation device, scaffolds may “off-load” the surgical repair and bear a large proportion of the load, especially during early phases of healing (313). Therefore, the maximum failure force of a scaffold is an important parameter to assess. X-Repair, LARS ligament, and Poly-Tape had the highest forces at failure. Previous studies using intact scaffolds found maximum failure forces of 796 ± 34 N for X-Repair (12), 998 ± 148 N for LARS ligament (314), and 780 ± 200 N for Poly-Tape (AKA Leeds-Keio) (315). While the absolute values are different, the relative differences between the three synthetic scaffolds are similar to our findings.

All three scaffolds have dense, aligned bundles of microscale synthetic fibres on the order of $20\text{-}30\mu\text{m}$ (316) (figures 2-3A-C) that provide high tensile

strength. The type of polymers from which the synthetic scaffolds are made can partially explain the variation in tensile strength between the synthetic scaffolds.

LARS ligament and Poly-Tape are made from polyethylene terephthalate (PET)(Table 2-1), which is relatively strong and stiff (317). X-Repair is made from poly-L-lactic acid (PLLA) and BioFiber is made from poly-4-hydroxybutyrate (PH4B). PLLA and PH4B have similar tensile strength, but PLLA is much stiffer than PH4B (318). The difference in strength between X-Repair and BioFiber is likely due to BioFiber having larger, fewer, and less aligned fibres. PET and PLLA have comparable tensile strength (319), so the similar results seen for X-Repair, LARS ligament, and Poly-Tape are supported by this.

The slight difference in the tensile failure force between LARS ligament and Poly-Tape is likely caused by variations in structure; while the fibre diameters are similar, LARS ligament has more fibres and they are more densely arrayed. Even though they are both made from PET, the molecular weight of a polymer can impact the mechanical properties of a scaffold (320) and may have varied between LARS ligament and Poly-Tape. However, this information is not readily available and was not determined experimentally, so it is difficult to reliably conclude what effect if any, this had.

GraftJacket, Permacol, and Conexa had substantially lower tensile failure forces than the synthetic scaffolds tested. We report no difference in tensile strength between the biological scaffolds. This contrasts with previous

studies that found differences between dermal scaffolds (195, 302). This may be due to similarities in scaffold thickness (Table 2-1), the selection of scaffolds tested (no previous study has compared GraftJacket, Permacol, and Conexa), in addition to the large differences required to reach statistical significance when comparing eight different materials.

No scaffold tested matched the tensile failure force of native tissue. Our values of 311 ± 41 N for maximum force at failure of supraspinatus tendon fell within the range of previously determined values for strips of human supraspinatus 80-652 N (10, 321). The wide range of reported values for supraspinatus tendon reiterates the need for intra-experimental comparisons.

The most common mode of rotator cuff re-tear following surgery is suture pull-out (200). X-Repair's suture pull-out force was significantly larger than all other scaffolds, except LARS ligament. X-Repair is a tightly woven mat of strong, synthetic fibres. Many of these fibres are loaded during suture pull-out, which helps to dissipate stress and reach higher failure forces. LARS ligament also contains many aligned, densely packed fibres, which provide high tensile strength. BioFiber, GraftJacket, Permacol, and Conexa performed similarly in suture pull-out. While previous studies have performed suture pull-out testing on scaffolds (12, 13, 199, 201), the variation in size and the type of suture technique used makes it difficult to directly compare results. This study was the first to directly compare the mechanical properties of seven commercially available scaffolds and compare them to the properties of

cadaveric human supraspinatus tendon. Again, no scaffold matched the properties of native tissue, for which suture failure preceded pull-through. The inability to reproduce the basic functional requirements of load bearing via sutures is of central importance to the success of scaffolds used in this role. Therefore, a reconsideration of scaffold design and/or application in the context of rotator cuff repair may be required.

The macroscale Young's modulus of LARS ligament and Poly-Tape were significantly higher than every other material tested. This is likely due to the fact that they are both made from PET, which is relatively stiff (317). All other materials had a similarly low Young's modulus, which was equivalent to the Young's modulus of supraspinatus tendon. While it is important for a repaired supraspinatus tendon to be stiff enough to effectively transfer forces, if it is too stiff, then it may increase the chance of re-injuring the tendon. Furthermore, the stiffness requirements of the scaffold will likely change over time, as native tissue invades and begins to replace the scaffolds. However, it is not clear how stiff the scaffold should be and how its stiffness should change as it degrades over time, in order to promote the optimal repair.

Results from macroscale rheometry testing showed that many scaffolds lacked the ability to dissipate energy during deformation. In general, the biological scaffolds had higher loss tangents than the synthetic scaffolds, most likely due to dissipation throughout the highly interconnected protein networks. This energy could, in theory, be transferred to cells in and around the scaffold and influence their behaviour. Fibroblasts respond to mechanical

stimulation through mechanotransduction pathways that are largely mediated through integrin-containing cell-matrix adhesion contacts (322). Indeed, tenocytes have been shown to upregulate ECM gene in response to increased cyclical shear stress (323). Thus, shear energy that is not dissipated by the scaffold could be transferred to the attached tenocytes, leading to an upregulation in ECM proteins. While some ECM upregulation would be favourable during the repair process, over-expression of ECM proteins could result in unwanted adhesions and excessive scar tissue formation.

The supraspinatus tendons had significantly higher loss tangent than every other material tested, suggesting that the tendon promotes a greater degree of energy loss in shear. High energy storage is vital for tendons such as the Achilles (324), but effective dissipation of energy in the scaffold is likely to play a role in protecting tendon tissue from stress concentration. In general, biological scaffolds had loss tangents most similar to the native supraspinatus tendon. This may be due to the extensive ECM protein networks seen in biological tissues that are able to dissipate energy more effectively than stiff synthetic polymers.

We also evaluated the mechanical properties of the scaffolds on the microscale, which can influence cell behaviour (234, 297-299). Specifically, fibroblasts respond to stiffer substrates by increasing their own stiffness (41), upregulating ECM genes (including collagen type III) (325), and increasing cell attachment and proliferation (326).

At the microscale, biological scaffolds were less stiff than synthetic scaffolds, and provided the best approximation to the properties of native tissue. The increased Young's modulus of Conexa relative to tendon, however, was unexpected and may be explained by differences in commercial processing techniques used. The similar microscale Young's modulus of Permacol compared with Conexa and GraftJacket was unexpected, because Permacol is cross-linked and cross-linking significantly increases microscale stiffness (327). This might be because Permacol is cross-linked with hexamethylene diisocyanate (HDMI); a relatively mild cross-linking agent (328). Of the synthetic scaffolds, only BioFiber had both a Young's modulus and loss tangent similar to that of tendon, which supports previous work showing that PH4B is relatively soft and elastic (318). The high Young's modulus of X-Repair, LARS ligament, and Poly-Tape is unsurprising because they are made from relatively stiff polymers. All synthetics with the exception of X-Repair had a similar loss tangent to that of native tissue.

Surface roughness can influence the mechanical properties of scaffolds (329, 330) and also affects cell adhesion (237), proliferation (40, 331, 332), gene expression (244), and morphology (331). X-Repair, LARS ligament, Poly-Tape, and Permacol had significantly increased surface roughness (of entire images) compared to BioFiber. This can be explained by differences in fibre curvature, with BioFiber having much greater fibre diameter and thus lower fibre curvature than X-Repair, LARS ligament, and Poly-Tape. Because roughness is measured by changes in probe height, variation in fibre curvature affects roughness measurements.

After accounting for fibre curvature, we found different results for surface roughness. Biological scaffolds were significantly rougher than synthetic scaffolds. The smooth and continuous surfaces of the synthetic scaffolds revealed few surface imperfections, through which cracks propagate during macroscale mechanical testing. However, it is unclear if such differences in roughness on the microscale significantly contributed to the observed differences in macroscale mechanical properties. Roughness could influence cell behaviour following *in vivo* implantation, which may alter the mechanical properties of the scaffold.

It is unreasonable to expect scaffold materials to match the complex structures of a highly specialised native tissue. However, close matching of microscale mechanical properties of scaffolds to those of native tissue holds promise for the application of scaffolds that will be implanted *in vivo*. In load bearing, the macroscale mechanical properties of scaffolds are defined by *force* rather than *stress* as geometry (specifically thickness) of the scaffolds is not strongly limiting. Combining scaffolds with closely matched microscale properties with materials that provide appropriate macroscale properties, or by simply increasing the thickness of existing scaffolds, it may be possible to create a scaffold with appropriate mechanical properties for rotator cuff repair.

For example, BioFiber possesses micro mechanical properties similar to supraspinatus tendon, but is much weaker on the macroscale. If the BioFiber fibres were smaller and densely arrayed, like those in X-Repair or LARS

ligament, then it may have been able to match the native tissue more accurately on both scales. Alternatively, synthetic scaffolds may be coated in a biological material, to combine macroscale strength with favourable microscale properties. Whether the addition of a single protein, such as collagen, to the surface of a synthetic scaffold significantly alters cell response remains unclear. However, efforts continue to elucidate important binding motifs in the ECM (such as RGD sequences) and greater understanding of these may help to determine the amount and type of cell-binding molecules that could be added to synthetic scaffolds to favourably influence cell response. It is clear that existing scaffolds do not specifically address the mechanical requirements of a rotator cuff repair and future scaffolds should be designed with these requirements in mind.

There are several limitations of this work. Firstly, we used an *in vitro* system that could not recapitulate the complex, cyclical loading of an *in vivo* environment. While our results reflect the mechanical properties of the scaffold immediately after implantation, the properties of each scaffold will change as it is remodelled and incorporated into the surrounding tissue. Secondly, we cut the scaffolds and supraspinatus tendon into relatively small pieces to standardise the tests and account for the limited amount of material available. The mechanical properties of smaller samples of scaffold may not precisely mimic the mechanical properties of the intact scaffold. Thirdly, we do not assess living tendon tissue and are thus unable to conclude that the mechanical properties of the cadaveric tendon pieces we used are representative of living tendon tissue.

Our study also presents several unique strengths. Drawing inter-experimental comparisons is difficult because studies often use different techniques and equipment, leading to a wide range of reported values (10, 321). Therefore, a primary strength of this study is that it is the first to directly compare the mechanical properties of commercial scaffolds with healthy supraspinatus tendon. Also, it is the first to analyse the micro mechanical properties of commercially available scaffolds.

2.7 Conclusions

On the macroscale, synthetic scaffolds, with dense arrays of aligned microscale fibres, demonstrated increased strength and greater suture-retention properties than biological scaffolds. Synthetic scaffolds with fewer, larger, and less aligned fibres were weaker on the macroscale.

On the microscale, biological scaffolds and synthetic scaffolds made from soft polymers were more similar to native supraspinatus tendon. However, no scaffold was able to approximate the mechanical properties of supraspinatus tendon on both the macroscale and microscale. Future work should focus on creating new scaffolds with desirable mechanical properties across different scales. Combining the macroscopic strength of synthetic scaffolds with the microscale properties of biological scaffolds would create a scaffold that more accurately approximates the properties of native tendon. Such a scaffold could improve the clinical outcome of patients undergoing surgical rotator cuff repair.

Chapter 3 : The response of tenocytes to rotator cuff scaffolds

3.1 Abstract

3.1.1 Background

Surgical repairs of torn rotator cuff tendons have high re-tear rates and many scaffolds have been developed to augment the repair. Understanding the interaction between patient-derived tenocytes and scaffolds is important for improving scaffold performance and tendon healing.

3.1.2 Materials and methods

In this *in vitro* study, we investigated the response of patient-derived tenocytes to eight different scaffolds: X-Repair, Poly-Tape, LARS Ligament, BioFiber (synthetic scaffolds), BioFiber-CM (biosynthetic scaffold), GraftJacket, Permacol, and Conexa (biological scaffolds). Cell attachment, proliferation, gene expression, and morphology were assessed.

3.1.3 Results

After one day, more cells attached to synthetic scaffolds with dense, fine and aligned fibres (X-Repair and Poly-Tape) than to other scaffolds tested. Despite low initial cell attachment, the human dermal scaffold (GraftJacket) promoted the greatest proliferation of cells over 13 days. Gene expression of *COL1A1* and *COL3A1* were upregulated in cells grown on non-cross-linked porcine

dermis (Conexa). Interestingly, the ratio of collagen I to collagen III mRNA was lower on all biological scaffolds compared to synthetic and biosynthetic scaffolds.

3.1.4 Conclusions

These findings demonstrate significant differences in the response of patient-derived tenocytes to scaffolds that are routinely used for rotator cuff surgery. Synthetic scaffolds promoted increased cell attachment and an elongated cellular phenotype, while biological scaffolds promoted cell proliferation and expression of collagen genes. However, no single scaffold was superior. Our results may help understand the way that patients' cells interact with scaffolds and guide the development of new scaffolds in the future.

3.2 Introduction

Scaffolds contain instructive cues that influence cell behaviour. Biological scaffolds are expected to retain biological (190, 333) and structural (334, 335) cues that instruct cell migration (336), proliferation (337), and phenotype (338) to promote repair (313, 339). The biological scaffolds used in this study were GraftJacket (human dermis), Permacol (cross-linked porcine dermis), and Conexa (non-cross-linked porcine dermis)(Table 2-1).

In contrast, synthetic scaffolds permit the customisation of specific mechanical (340) and chemical properties (98) that can stabilise the repair (207). Several synthetic scaffolds were used in this study: X-Repair (poly-l-lactic acid), Poly-Tape (polyethylene terephthalate), LARS ligament (polyethylene terephthalate), and BioFiber (poly-4-hydroxybutyrate)(Table 2-1). Biosynthetic scaffolds can include native signalling cues from biological and synthetic scaffolds. This study used BioFiber-CM (poly-4-hydroxybutyrate coated in bovine collagen) as a biosynthetic scaffold.

There is also an increasing appreciation that the microscale surface properties of scaffolds, such as stiffness (234), roughness (238), and morphology (242) can influence cell behaviour. Thus, it is important to appreciate the structure and form of scaffold surfaces when evaluating cell behaviour.

Pre-clinical studies have reported favourable results using both biological (341, 342) and synthetic scaffolds (12, 207) for rotator cuff repair. However, results have not always been replicated in human *in vivo* studies

and several clinical studies have reported adverse reactions (343, 344) and increased failure rates (296, 345) following scaffold use. This discrepancy suggests inadequacy of the existing pre-clinical models to replicate human pathology. Many studies artificially lacerate tendons in healthy animals (213, 214) that undergo spontaneous repair in the absence of scaffold or use healthy tenocytes for *in vitro* testing (341, 342). Previous studies have demonstrated differences in tenocytes derived from healthy tissue compared to those derived from diseased tissue: specifically, differences in the expression of collagen (346), inflammatory mediators (126), and apoptotic markers (347). Previous studies that have investigated the response of tenocytes to biomaterials have overlooked this distinction.

Thus, the precise manner by which patients' cells interact with these scaffolds remains unclear. Tenocytes are the most abundant cell type in tendons and will experience many interactions with any implanted scaffold. Therefore, it is important to understand how patient-derived tenocytes interact with existing scaffolds and new scaffolds under development. In addition, the large number of scaffolds and variety of experimental paradigms used to test scaffolds *in vitro* makes it difficult to compare scaffolds and assess their potential for use *in vivo*.

3.3 Aims and hypotheses

Therefore, the primary purpose of this *in vitro* study was to compare the response of tenocytes from rotator cuff tear patients (cells derived from n=3 patients) to eight commercially available scaffolds in terms of initial cell attachment, cell proliferation, final cell number, gene expression, and cell morphology. The secondary purpose of the study was to evaluate the form and structure of the scaffold surfaces, with a particular focus on the fibre diameter of the synthetic scaffolds.

It was hypothesised that cell attachment, proliferation, and gene expression would be greater on biological scaffolds compared to synthetic scaffolds. Due to the maintenance of biological and structural cues, it was also hypothesised, that biological scaffolds would promote more elongated bi-directional tenocyte morphology with greater connectivity, compared to synthetic scaffolds.

3.4 Materials and methods

3.4.1 Scaffold selection

Scaffolds were selected based on presence in the literature and commercial availability (Table 3-1). Biological, biosynthetic, and synthetic scaffolds were selected to represent a cross-section of the spectrum of available scaffolds indicated for rotator cuff repair.

Table 3-1. Information on each of the scaffolds used.

Scaffold Name	Manufacturer	Industrial Supplier	Source	Scaffold Type	Cross-Linked	Terminally Sterilized
X-Repair	Synthasome	Synthasome	Poly- L-lactic-acid	Synthetic	N	Y
LARS Ligament	LARS	LARS	Polyethylene Terephthalate	Synthetic	N	Y
Poly-Tape	Xiros plc, Neoligaments	Neoligaments	Polyethylene Terephthalate	Synthetic	N	Y
BioFiber	Tornier	Tornier	Poly(4-hydroxybutyrate)	Synthetic	N	Y
BioFiber-CM	Tornier	Tornier	Poly(4-hydroxybutyrate) + Bovine Collagen	Biosynthetic	N	Y
GraftJacket	LifeCell	Wright Medical Technology	Human Dermis	Biological	N	N
Permacol	Tissue Science Laboratories	Zimmer	Porcine Dermis	Biological	Y	Y
Conexa	Tornier	Tornier	Porcine Dermis	Biological	N	Y

3.4.2 Human tendon tissue acquisition

Biopsies of torn supraspinatus tendons were taken from three male patients aged between 40 and 65 years ($\pm$ 13.05 years SD) undergoing surgical repair for large or massive rotator cuff tears. Tendon biopsies were collected with fully informed donor consent, according to the Declaration of Helsinki, under ethics from the Oxford Musculoskeletal Biobank (09/H0606/11), in compliance with the National and Institutional ethical requirements. During surgery, biopsies were immediately transferred into sterile tubes containing DMEM/F12 media (Lonza, Slough, UK) and were then used for cell isolation within X hours of surgery.

3.4.3 Tenocyte cell isolation and culture

Tenocytes were isolated by cutting the tissue biopsies into small pieces, approximately 2mm x 2mm square and transferred to 6-well plates (Corning Inc., Corning, NY, USA). Explants were initially cultured in media containing DMEM/F12 media (Lonza, Slough, UK), 50% fetal bovine serum (Labtech, Uckfield, UK), and 1% Penicillin-Streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). After 1 week, cells were then cultured with supplemented medium: DMEM/F12 media (Lonza, Slough, UK), 10% fetal bovine serum (Labtech, Uckfield, UK), and 1% Penicillin-Streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). Supplemented media was replaced every 3 days. Once cells had reached confluence, they were scraped and transferred to 10cm petri dishes (Greiner, Frickenhausen, Germany). Cells from each patient were used separately for each experiment and were

used at passage 3 to avoid phenotypic drift experienced at higher passages (348).

3.4.4 Scaffold preparation and tissue culture

Scaffolds were prepared according to manufacturers' instructions, then conditioned in supplemented media for 24 hours prior to cell seeding in static culture. Cells were seeded at a density of 100,000 cells/cm² for all experiments, except for cell morphology, where cells were seeded at a density of 10,000 cells/cm². After cell seeding, all scaffolds were transferred to fresh 24-well plates (48-well plates for the morphology study). Scaffolds were then transferred to new 24-well plates (48-well plates for the morphology study) with fresh media every 3 days such that only cells attached to the scaffolds were accounted for in viability assays. Unless stated otherwise, tissue culture plastic (TCP) was used as a positive control and unseeded (cell free) scaffolds were used as negative controls.

3.4.5 Environmental scanning electron microscopy (ESEM)

A 0.25cm² piece of each scaffold was rinsed in phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, MO USA) for 10 minutes then fixed in 2.5% glutaraldehyde (Sigma-Aldrich, St. Louis, MO USA) for 24 hours. Scaffolds were rinsed in deionised water before undergoing a graded ethanol series (Sigma-Aldrich, St. Louis, MO USA) (40%, 70%, 90%, 95% and 100% each for 10 minutes). Next, 100µl of hexamethyldisilazane (Alfa Aesar, Ward Hill, MA, USA) was added to each scaffold for 24 hours. Scaffolds were gold coated using a SC7620 Mini Sputter Coater System (Quorum Technologies Ltd,

Laughton, UK) and images were taken at 50X magnification using an Evo LS15 Variable Pressure Scanning Electron Microscope (Carl Zeiss AG, Oberkochen, Germany). The diameter of individual fibres was determined using ImageJ (National Institute of Health, Bethesda, MD, USA).

3.4.6 Initial cell attachment and final cell number

Six 1cm² samples of each scaffold were seeded with 100,000 cells in a 24-well plate (Corning Inc., Corning, NY, USA). Three scaffolds were processed on day 1 to measure initial cell attachment. On day 13, the remaining scaffolds were processed to assess the final cell number.

For quantification of DNA, a surrogate measure for cell number, scaffolds were rinsed twice in PBS (Sigma-Aldrich) then suspended in 350µl of 0.2% Nonidet P40 (Fluka Biochemika, Buchs, Switzerland). Scaffolds were freeze-thawed three times and sonicated twice for 10 seconds using an ultrasonic cell disruptor (Misonix, Farmingdale, NY, USA). The double stranded DNA (dsDNA) content of each well was quantified using the Quant-iT PicoGreen assay (Invitrogen, Paisley, UK). Briefly, 50µl of each sample was added to 50µl of 1X Tris-EDTA (TE) buffer, and 100µl of 1X PicoGreen reagent to make a reaction volume of 200µl.

Each reaction was performed in duplicate, for which a mean value was calculated. A standard curve (1000ng/ml, 100ng/ml, 10ng/ml, 1ng/ml, and 0ng/ml) was created using the DNA standard provided in the kit. The test was performed in a 96-well fluorescence plate (Greiner) and the plate was covered in foil to reduce photo bleaching, and incubated for 5 minutes at

room temperature. Fluorescence was then measured at 480nm excitation and 520nm emission using a FLUOstar optima microplate reader (BMG Labtech, Ortenberg, Germany).

3.4.7 Cell proliferation

Six 1cm² samples of each scaffold were seeded with 100,000 cells and cultured in 24-well plates (Corning). A calibration curve was generated by seeding cells at the following densities in a 48-well plate (Corning Inc., Corning, NY, USA): 5x10³/cm², 1x10⁴/cm², 5x10⁴/cm² and 1x10⁵/cm². A 48-well plate was used as a control because the surface area is approximately 1cm². The calibration curve was used to approximate cell number as represented by AlamarBlue metabolic activity measurements, throughout the experiment. The numbers of cells on scaffolds were quantified using AlamarBlue Assay, which has been validated previously (349, 350). Every three days, scaffolds were incubated in 5% AlamarBlue (Invitrogen, Paisley, UK) in supplemented media for 2 hours. Fluorescence was measured at 485nm excitation and 520nm emission using a FLUOstar optima microplate reader (BMG Labtech, Ortenberg, Germany).

AlamarBlue was used to measure cell viability in a continuous fashion, whereas PicoGreen was used to support AlamarBlue data, but PicoGreen could not be evaluated continuously because it requires sample destruction.

3.4.8 Gene expression

Three 1cm² samples of each scaffold were seeded with 100,000 cells in a 24-well plate (Corning). After 13 days, scaffolds were rinsed in PBS (Sigma-

Aldrich) then fixed in 1ml TRIzol (Invitrogen, Paisley, UK). mRNA was extracted using the Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA, USA) and mRNA quality was checked using a Nanodrop ND1000 (Nanodrop Technologies, Willington, DE, USA). All samples had 260/280 value greater than 1.6. Approximately 1000ng of mRNA was reverse transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) in a 20 μ l reaction. For RT-qPCR final cDNA was diluted to 5ng/ μ l and 1 μ l was used in a 10 μ l reaction with Fast SYBR Master Mix (Applied Biosystems, Waltham, MA, USA) according to manufacturer's protocol. The RT-qPCR reaction was performed using a ViiA7 real-time PCR machine (Applied Biosystems, Waltham, MA, USA). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and TATA-binding protein (*TBP*) were used as endogenous reference genes and gene expression was calculated using the comparative CT method (351). QuantiTect primers for *COL1A1*, *COL3A1*, *COL2A1*, *COL6A1*, *TNC*, *DCN*, *SCX-B*, *TNMD*, *ACAN*, *VCAN*, *GAPDH* and *TBP* were used for RT-qPCR (Qiagen, Hilden, Germany).

3.4.9 Cell morphology

Nine 0.5cm x 0.5 cm samples of each scaffold were seeded with 2.5×10^4 cells in a 48-well plate (Corning). Three samples of each scaffold were fixed in 10% formalin (Fischer Scientific, Loughborough, UK) on days 1, 7, and 13. Samples were incubated in 1ml 0.5% Triton-X (Thermo Fisher Scientific, Waltham, MA, USA) for 10 minutes, then rinsed in PBS (Sigma-Aldrich). A 100nM solution of acti-stain 488 phalloidin (Cytoskeleton Inc., Denver, CO, USA) and 4', 6-Diamidino-2-Phenylindole (DAPI) (Thermo Fisher Scientific, Waltham,

MA, USA) was generated in PBS (Sigma-Aldrich) and 500µl of the solution was added to each scaffold and incubated for 2 hours in the dark at 4°C. Scaffolds were rinsed in PBS (Sigma-Aldrich) and images were captured at 40X magnification using a Zeiss LSM710 confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

3.4.10 Statistical analysis

A Kolomogorov-Smirnov test was performed for each assay to determine if data were normally distributed. Technical replicates were included for assays where the number of biological replicates was not sufficient to determine normality. Unless otherwise stated, a one-way ANOVA was performed and a Tukey post-hoc test was completed to compare all scaffolds tested. To compare the mRNA expression ratio of *COL1A1* and *COL3A1* between dermal and synthetic/biosynthetic scaffolds, a two-tailed unpaired t-test was performed. Statistical analysis was performed using GraphPad PRISM (GraphPad Software Inc., La Jolla, CA, USA).

Statistical significance was considered at * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$ and 95% confidence intervals (CIs) of minimum difference were reported. Statistical significance was presented as the maximum statistical differences between the designated scaffold and every other scaffold (the highest p-value where there were multiple being considered). For example, if the difference between X-Repair and BioFiber is $p \leq 0.01$ but the difference between X-Repair and Poly-Tape is $p \leq 0.05$, then the difference

between X-Repair and, BioFiber and Poly-Tape will be represented as $p \leq 0.05$, because this is the maximum p-value between both.

3.5 Results

3.5.1 Scaffold morphology

Synthetic scaffolds demonstrated different weave patterns (figures 3-1A-D) and fibre diameters (figure 3-1I). The average fibre diameter of BioFiber was larger than the three other synthetic scaffolds ($p \leq 0.001$, 95% CI: 158, 163)(figure 3-1I). The average fibre diameter of LARS Ligament was larger than X-Repair ($p \leq 0.01$, 95% CI: 0.86, 5.95) and and Poly-Tape ($p \leq 0.001$, 95% CI: 2.27, 7.36) respectively, while the average fibre diameters of X-Repair and Poly-Tape were equivalent (figure 3-1I).

X-Repair (figure 3-1A) and Poly-Tape (figure 3-1B) had plain weave patterns, with straight and perpendicular bundles of fibres. LARS ligament (figure 3-1C) had an array of straight fibres with the addition of twisted fibre bundles. BioFiber (figure 3-1D) had a lattice of parallel fibres, interwoven with curved fibres. BioFiber (figure 3-1D) displayed thick fibres with high porosity, whereas BioFiber-CM (its bovine-collagen coated equivalent—figure 3-1E) appeared flat and dense. GraftJacket (figure 3-1F), the human dermal scaffold, appeared rougher than the two porcine dermal scaffolds of Permacol (figure 3-1G) and Conexa (figure 3-1H).

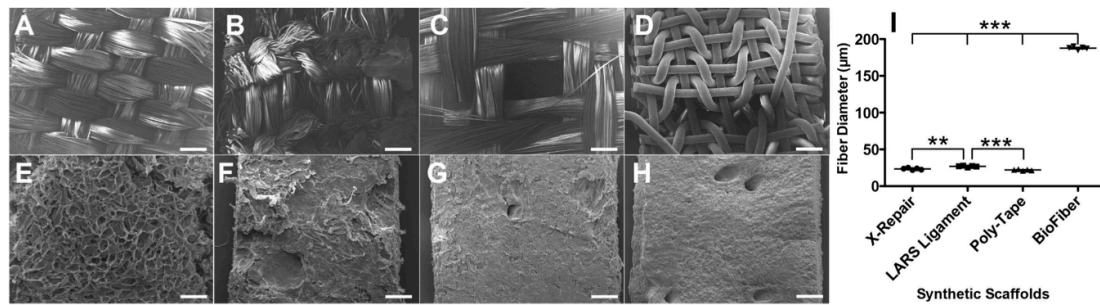


Figure 3-1. Scaffold morphology.

ESEM micrographs showing the appearance of the scaffolds. X-Repair (A), LARS Ligament (B), Poly-Tape (C), BioFiber (D), BioFiber-CM (E), GraftJacket (F), Permacol (G), Conexa (H). 50X magnification. Scale bar=600μm. The diameter of individual fibres of the synthetic scaffolds was measured from the ESEM images (I). Bar represents mean. ** $p \leq 0.01$ and *** $p \leq 0.001$.

3.5.2 Cell attachment and proliferation

After 1 day, more cells attached to X-Repair ($p \leq 0.05$, 95% CI: 5, 81) and Poly-Tape ($p \leq 0.01$, 95% CI: 12, 88) than any other scaffold as measured by dsDNA content (figure 3-2A). All other scaffolds demonstrated similar amounts of dsDNA. Figures 3-2A and 3-2B represent boxplots.

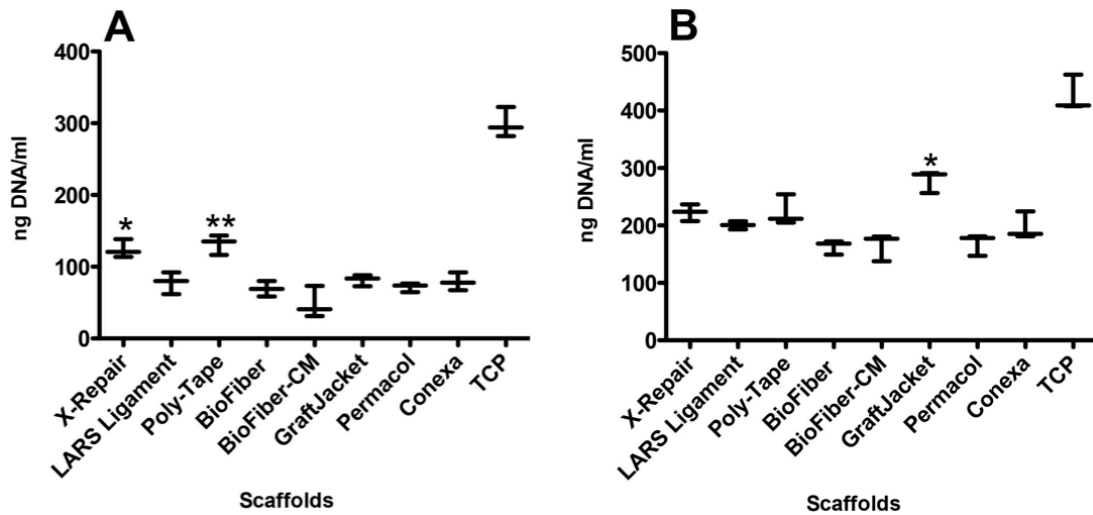


Figure 3-2. Amount of DNA extracted from cells on scaffolds on day 1 (A) and day 13 (B).

DNA was quantified using PicoGreen and this was used to approximate the number of cells that attached to each scaffold on day 1 (A) and day 13 (B). On day 1, X-Repair had significantly more DNA than every other scaffold ($p \leq 0.05$) except Poly-Tape (A). On day 1, Poly-Tape had significantly more DNA than every other scaffold ($p \leq 0.01$) except X-Repair (A). On day 1, there was no statistical difference in DNA quantity between X-Repair and Poly-Tape (A). On day 13, GraftJacket had more DNA than every other scaffold ($p \leq 0.05$) (B). Graphs represent boxplots with median, minimum and maximum ($n=3$).

AlamarBlue was used to study the change in cell viability (which was used as a further surrogate for cell number) on the scaffolds over 13 days. After 1 day, cell number/cm² was higher on X-Repair ($p \leq 0.05$, 95% CI: 1174, 18378) and Poly-Tape ($p \leq 0.05$, 95% CI: 147, 17352) than any other scaffold (figure 3-3A). After 13 days, cell number/cm² was higher on GraftJacket ($p \leq 0.05$, 95% CI: 4038, 68229) than any other scaffold (figure 3-3A). These results were confirmed using the PicoGreen assay to measure dsDNA content on day 1 (figure 3-2A) and day 13 (figure 3-2B). While AlamarBlue measures cell viability in a continuous way, PicoGreen measures dsDNA content in a destructive manner. Even though cell viability and dsDNA content are

related, they are distinct parameters that are complementary, but not interchangeable. No statistical differences in cell number/cm² were observed between any other scaffolds (figure 3-3A).

For AlamarBlue assays cell number per/cm² for each scaffold was standardised to day 1 values to determine the relative proliferation of cells that attached to each scaffold (figure 3-3B). Of the cells that initially attached, greater proliferation was seen on GraftJacket compared to every other scaffold after 7 days ($p \leq 0.05$, 95% CI: 0.32, 3.20), 10 days ($p \leq 0.05$, 95% CI: 0.19, 4.93) and 13 days ($p \leq 0.05$, 95% CI: 0.78, 7.27) (figure 3-3B). There were no statistically significant differences at any other time points.

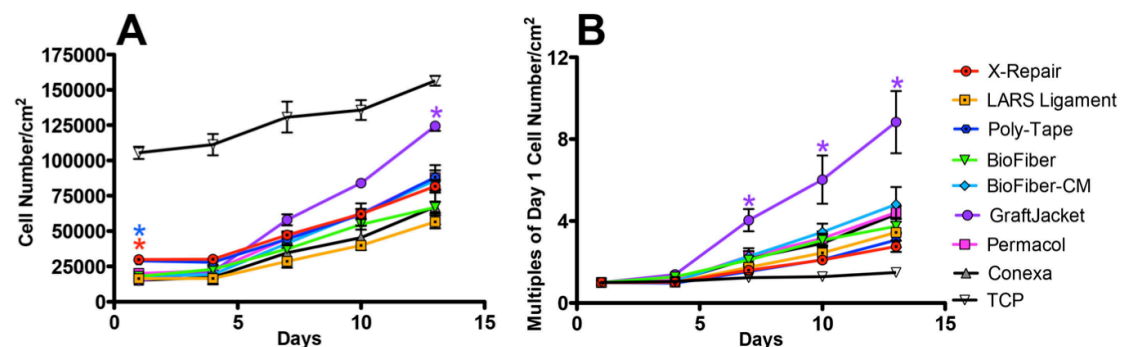


Figure 3-3. Proliferation of cells on scaffolds over 13 days.

The absolute proliferation of cells on scaffolds was measured every 3 days for 13 days using AlamarBlue (A). On day 1, more cells were attached to X-Repair than every other scaffold ($p \leq 0.05$) except Poly-Tape (A). On day 1, more cells attached to Poly-Tape than every other scaffold ($p \leq 0.05$) except X-Repair (A). On day 1, there was no statistical difference in cell number between X-Repair and Poly-Tape (A). On day 13, more cells attached to GraftJacket than every other scaffold ($p \leq 0.05$) (A). Measurements were also standardized to day 1 to determine the relative proliferation of the cells that did attach (B). Of the cells that did attach, cells were significantly more proliferative on GraftJacket on day 7 ($p \leq 0.05$), day 10 ($p \leq 0.05$), and day 13 ($p \leq 0.05$) when compared to every other scaffold (B). Graphs represent median with minimum and maximum (n=3).

3.5.3 Gene expression

After 13 days, RT-qPCR was performed to determine whether mRNA expression of collagen types I (*COL1A1*) and III (*COL3A1*) varied between scaffolds. Expression of collagen type I (*COL1A1*) and III (*COL3A1*) were higher in cells on Conexa than all other scaffolds (*COL1A1* → $p \leq 0.05$, 95% CI: 0.013, 0.54. *COL3A1* → $p \leq 0.05$, 95% CI: 0.010, 2.02)(figure 3-4A and 3-4B). The ratio of *COL1A1*:*COL3A1* mRNA expression was higher on synthetic and biosynthetic scaffolds (X-Repair, LARS Ligament, Poly-Tape, BioFiber, BioFiber-CM) than dermal scaffolds (GraftJacket, Permacol, Conexa) ($p=0.0009$) (figure 3-4D). Figures 3-4 A-D and 3-5 A-H are boxplots.

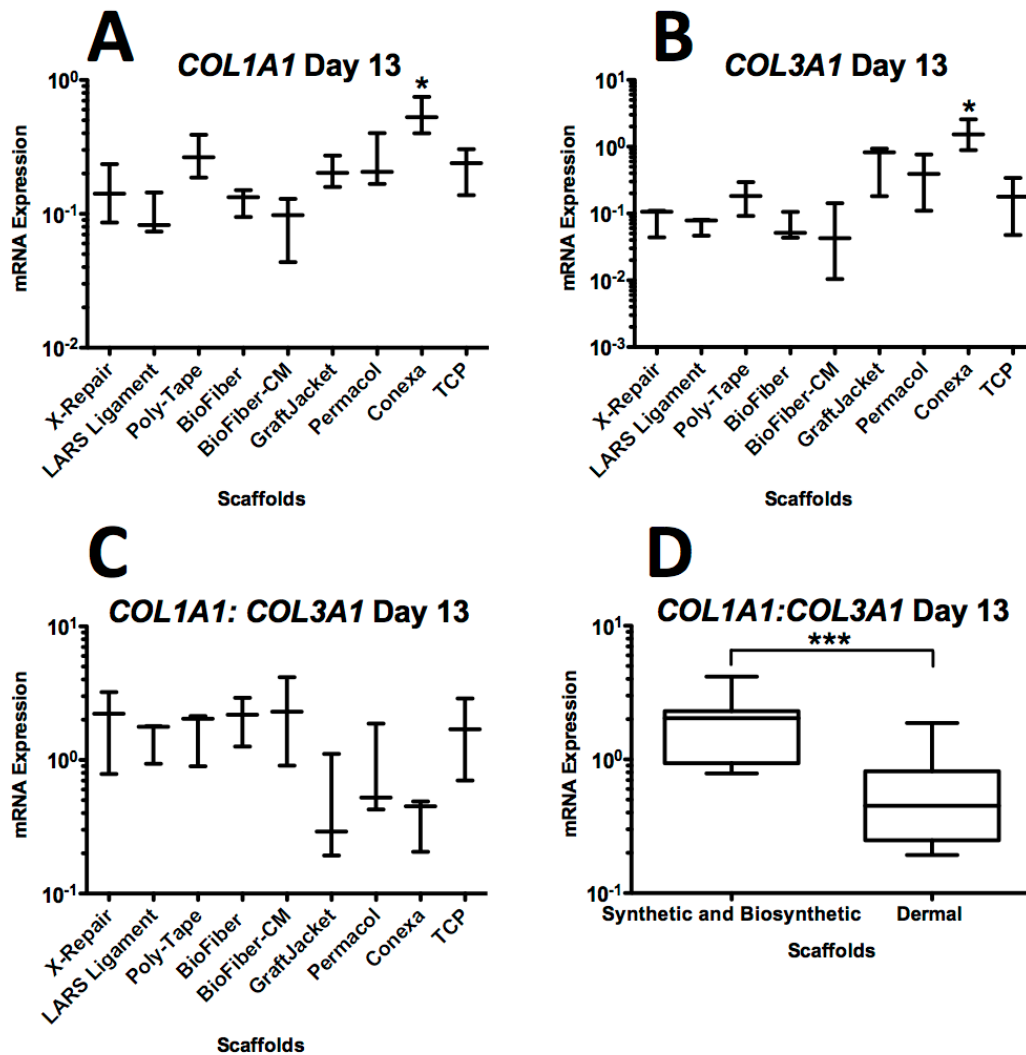


Figure 3-4. Day 13 mRNA expression.

After 13 days, mRNA expression of *COL1A1*(A) and *COL3A1*(B) was calculated relative to *GAPDH*. After 13 days, *COL1A1* was greater on Conexa than every other scaffold ($p \leq 0.05$)(A). This is shown in figure 3-4A as one asterisk above Conexa. After 13 days, *COL3A1* was greater on Conexa than every other scaffold ($p \leq 0.05$)(B). This is shown in figure 3-4B as one asterisk above Conexa. The ratio of *COL1A1*:*COL3A1* mRNA after 13 days was also calculated (C). An unpaired t-test was performed to compare the ratio of *COL1A1*:*COL3A1* on synthetic and biosynthetic scaffolds, against dermal scaffolds (D). The ratio of *COL1A1*:*COL3A1* was higher on synthetic and biosynthetic scaffolds, compared to dermal scaffolds ($p = 0.0009$) (D). This is shown in figure 3-4D as three asterisks above line comparing the two groups. Figures 3-4 A-D are boxplots, which represent median with minimum and maximum values ($n = 3$).

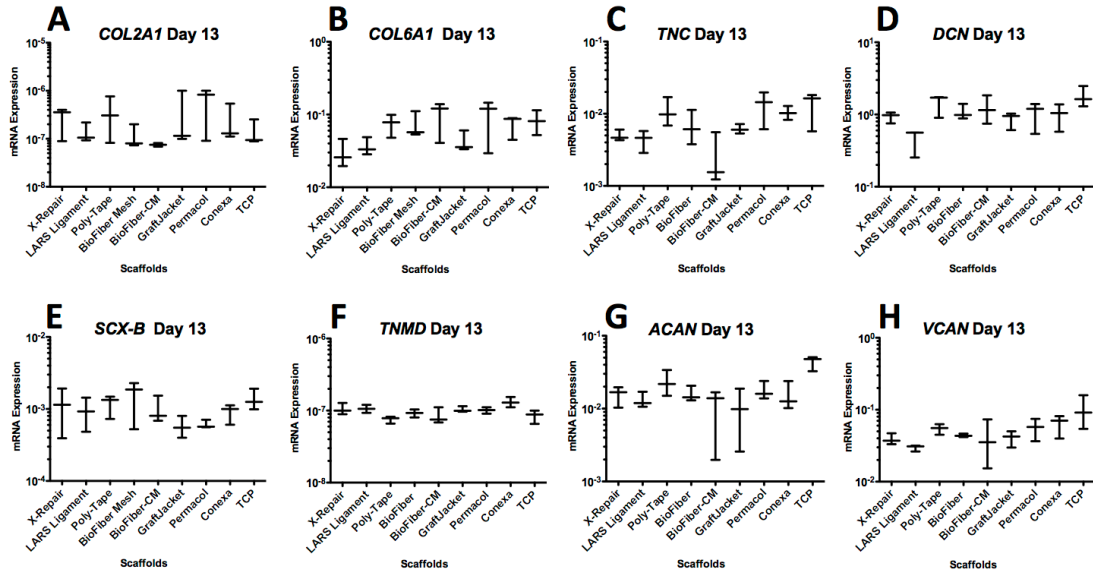


Figure 3-5. Day 13 mRNA expression.

After 13 days, mRNA expression was calculated relative to *GAPDH*. *COL2A1* (A), *COL6A1* (B), *TNC* (C), *DCN* (D), *SCX-B* (E), *TNMD* (F), *ACAN* (G), *VCAN* (H). None of the scaffolds demonstrated significant differences for gene expression. Figures A-H are boxplots that represent median with minimum and maximum values (n=3).

3.5.4 Cell morphology

On day 13, cells on X-Repair, LARS ligament, and Poly-Tape appeared morphologically similar to healthy native tenocytes, with extended lamellipodia aligning along the lengths of the scaffold fibres (figure 3-6C, F, and I respectively). On day 13, cells on BioFiber aligned along fibres, but also exhibited lamellipodia that were transverse to fibre direction (figure 3-6L). In contrast, on day 13 cells on BioFiber-CM, GraftJacket, Permacol, and Conexa exhibited filopodia that extended in different directions (figure 3-6O, R, U, and X respectively). After 13 days, cells on GraftJacket were smooth (figure 3-6R), while cells on Permacol (figure 3-6U) and Conexa (figure 3-6X) appeared dendritic and punctate.

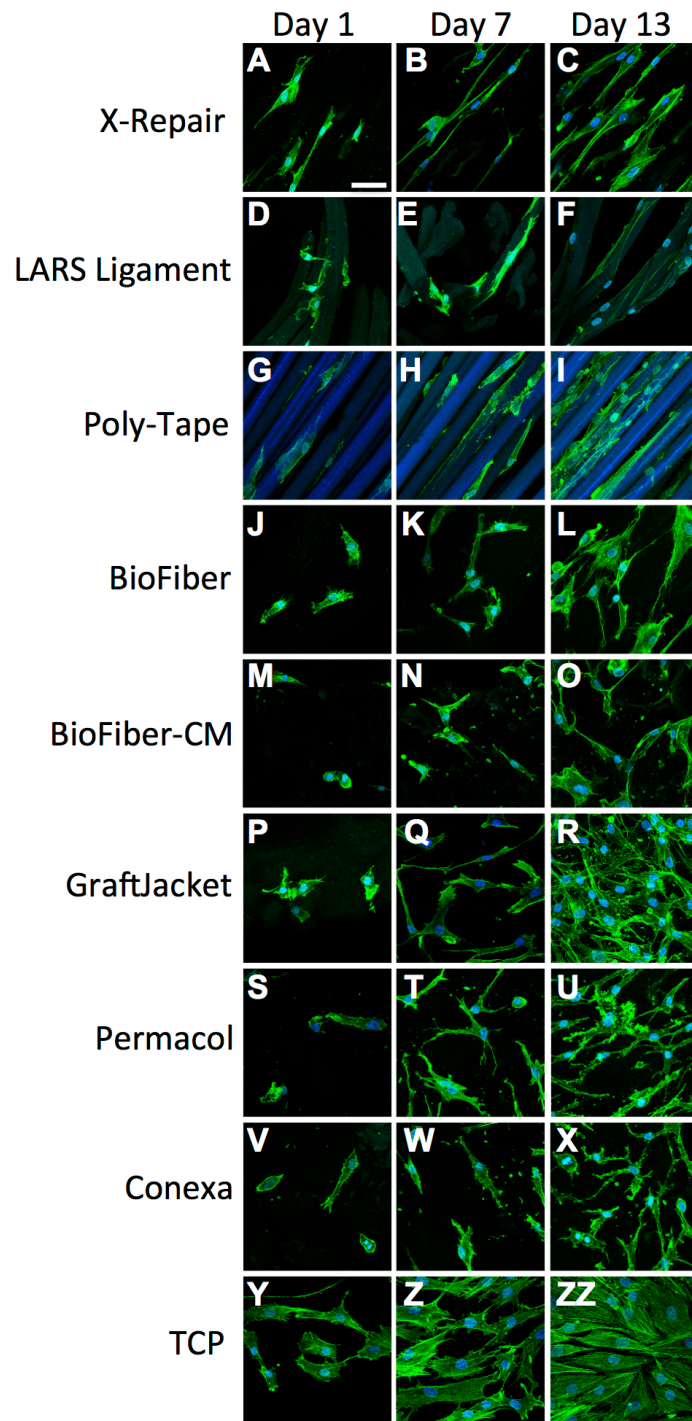


Figure 3-6. Fluorescent images of cell morphology on scaffolds.

Cell morphology was assessed on days 1, 7, and 13 using confocal microscopy. Cell cytoskeleton was stained with phalloidin (green) and nucleus was counterstained using DAPI (blue). Magnification 40X. Scale bar=50 μ m. X-Repair (A, B, C), LARS Ligament (D, E, F), Poly-Tape (G, H, I), BioFiber (J, K, L), BioFiber-CM (M, N, O), GraftJacket (P, Q, R), Permacol (S, T, U), Conexa (V, W, X), TCP (Y, Z, ZZ).

3.6 Discussion

Rotator cuff tears are a prevalent pathology, and surgical repair is not always effective. Numerous scaffolds have been developed to augment the surgical repair and reduce re-tearing. However, little is known about how native tenocytes interact with these scaffolds and how this interaction influences the healing process. In this study, we compared the response of patient-derived rotator cuff tenocytes to eight commercially available biological, biosynthetic, and synthetic scaffolds. Results from the study show that X-Repair and Poly-Tape (synthetic scaffolds) promoted the greatest cell attachment after 1 day and that GraftJacket (biological) promoted the greatest level of cell proliferation over 13 days. Expression of *COL1A1* and *COL3A1* was significantly upregulated in cells grown on Conexa (biological) after 13 days. In addition, the ratio of *COL1A1* to *COL3A1* mRNA was lower on all biological scaffolds compared to synthetic and biosynthetic scaffolds.

The interaction of cells with a scaffold is often mediated by a layer of proteins adsorbed to the scaffold surface, known as the provisional matrix. The provisional matrix is formed when serum proteins (from media *in vitro* or blood *in vivo*) adsorb to the scaffold surface (352). The type and orientation of the proteins in the provisional matrix depends largely upon the chemical and physical properties of the scaffold (353). Also, the provisional matrix changes over time due to the Vroman effect (354), whereby proteins adsorb and desorb based largely upon their size and thermodynamic properties. In this study, all scaffolds were pre-conditioned in media with 10%FBS overnight prior to cell seeding. While each scaffold received the same treatment, it is

likely that the type of provisional matrix that formed differed between the scaffolds tested. It is unclear whether the provisional matrix on each scaffold changed significantly during the pre-conditioning period. However, it is likely that the provisional matrix influenced the cell responses observed in this study.

For an implanted scaffold to be incorporated into the body, native cells must attach to its surface and migrate into the material. For adherent cells such as tenocytes, attachment is necessary for proliferation (355) and survival (356, 357). After 1 day, cell attachment was greatest on X-Repair and Poly-Tape, both synthetic scaffolds with dense arrays of aligned fibres with diameters of $\sim 22\text{-}23\mu\text{m}$ (figure 3-1I). This result was unexpected as cells usually adhere more to hydrophilic surfaces and less to hydrophobic surfaces (358-361). X-Repair and Poly-Tape are composed of poly-L-lactic acid polyethylene terephthalate respectively, both of which are hydrophobic (362, 363). In contrast, GraftJacket, Permacol, and Conexa are derived from mammalian dermis which is hydrophilic (364).

One plausible explanation is that synthetic micro-fibre scaffolds provide a larger effective surface area than biological scaffolds, allowing cells to attach above and within the scaffold. Previous studies have demonstrated increased cell attachment to biomaterials with increasing surface area (365-367). Furthermore, the quantity and contents of the provisional matrix likely varied between scaffolds, which could also help explain differences in cell attachment.

Surface stiffness may also affect cell attachment. At the microscale, X-Repair and Poly-Tape are stiffer than many of the other scaffolds (Figure 2-4B), and previous work has demonstrated preferential attachment of fibroblasts to stiffer surfaces (227, 326). While reduced stiffness of BioFiber, GraftJacket, Permacol, and Conexa may help explain reduced cell attachment, stiffness does not adequately explain why LARS ligament, a scaffold with high surface stiffness, demonstrated reduced cell attachment.

Fibroblasts attach more readily to smoother surfaces than rougher surfaces (235, 236). These previous studies partially support the results presented here; X-Repair and Poly-Tape both have reduced roughness (after accounting for fibre curvature, figure 2-6B) compared to the biological scaffolds, and both demonstrated higher initial cell attachment. However, roughness does not sufficiently explain why LARS ligament and BioFiber (synthetic scaffolds also with reduced roughness) did not also exhibit high initial cell attachment. It appears that a variety of scaffold factors influence cell attachment.

After 13 days, cell proliferation was greatest on GraftJacket, a minimally processed human dermis composed of collagen types I, III, IV and VII, elastin, proteoglycans and native growth factors, such as Fibroblast Growth Factor (FGF) (368). There are 18 members in the FGF family and their influence on cell proliferation has been well documented (369, 370). The presence of FGF in combination with minimally processed ECM could explain

the increased tenocyte proliferation seen on GraftJacket—however, this hypothesis remains to be investigated.

While our study suggests that human cells proliferate more on human dermis than porcine dermis, it is unclear whether this response is due to difference in tissue source (human vs. porcine) or processing techniques used during manufacturing. GraftJacket is derived from human dermis that is not sterilised or cross-linked (342) and is classified as human tissue for transplantation (179) under the FDA Code of Federal regulations Title 21 Part 1270 (310). In contrast, Permacol is derived from porcine dermis and undergoes extensive mechanical and enzymatic processing (179, 371) and is chemically cross-linked (195). Conexa is also derived from porcine dermis and is processed to minimise immunological reactions, but is not cross-linked (310).

The proprietary nature of these processing techniques makes it difficult to determine their precise impact on the ECM and the subsequent tenocyte response. While several studies have investigated the effect of these processing techniques (372, 373) on cellular response, only a small number of techniques were compared and therefore future investigations are required

Despite increased cell attachment on X-Repair and Poly-Tape, there was no corresponding significant increase in cell proliferation over 13 days. This result is supported by previous studies, which have shown that surface area is correlated with increased cell attachment (365-367) but not necessarily with increased cellular proliferation (367).

However, previous work has also demonstrated increased fibroblast proliferation on stiffer materials (374, 375), which we did not observe. In fact, when standardised to initial cell attachment (figure 3-3B), there was a trend for the biological scaffolds (BioFiber-CM, GraftJacket, Permacol, Conexa), which had reduced surface stiffness (figure 2-4B), to promote more proliferation over 13 days compared to the synthetic scaffolds (X-Repair, LARS ligament, Poly-Tape, and BioFiber). Thus, the presence of native ECM proteins and/or growth factors may override the influence that surface stiffness may have on cell proliferation. Surface roughness may also play a role, with increased surface roughness being correlated with increased cell proliferation (40). Increased roughness promotes the adsorption of serum proteins, which subsequently increases cell attachment and proliferation (376). Furthermore, fibroblasts reduce proliferation when contact is made with neighbouring cell (contact inhibition) (377, 378). Rougher surfaces, such as those seen in the biological scaffolds (figure 2-6B), may provide a physical barrier that reduces cell-cell interactions and reduces the effect of contact inhibition. However, future work is required to explore this hypothesis further.

After 13 days, expression of both *COL1A1* and *COL3A1* was significantly upregulated in cells on Conexa. Cellular production of new extracellular matrix proteins is critical for healing and neo-tissue formation. Collagen type I is the fundamental component of tendon (379, 380) and affords the tendon with high strength (381). In contrast, collagen III is associated with tendon healing and fibrosis (382, 383) and is mechanically

inferior to collagen I (346). While no individual scaffold demonstrated a statistically different ratio of *COL1A1:COL3A1*, there was a clear difference between dermal scaffolds (GraftJacket, Permacol, Conexa) in comparison to biosynthetic (BioFiber-CM) and synthetic scaffolds (X-Repair, LARS Ligament, Poly-Tape, BioFiber).

When stratified into separate groups, dermal scaffolds demonstrated a lower *COL1A1:COL3A1* ratio than biosynthetic and synthetic scaffolds. Lower ratios of collagen I to collagen III results in thinner collagen fibres (384) and reduced tissue stability (385, 386). Decreased ratios of collagen I to collagen III expression is seen during skin scarring (386) and it is interesting to consider the possibility that residual biophysical and biochemical cues in the dermal scaffolds promote this behaviour.

Interestingly, Conexa also has a greater surface stiffness than GraftJacket and Permacol (figure 2-4B) and fibroblasts respond to stiffer substrates by increasing expression of ECM genes, including collagen type III (325). In addition, native growth factors, such as TGF- β can influence collagen expression (387), and their residual presence in the biological scaffolds may help explain the observed results. GraftJacket contains FGF, but not TGF- β or VEGF (388), which correlated well with increased cell proliferation and reduced collagen expression. However, this study did not evaluate growth factor content in any of the scaffolds, so we are unable to definitely determine what effect, if any, they may have had.

Previous work has shown that increased tenocyte proliferation on collagen scaffolds can improve their mechanical properties (389) and it is possible that increased collagen deposition translates to improved macro mechanical properties. The number of cells and the amount of collagen each cell secretes could influence this. A preliminary study found that the presence of cells over 28 days *in vitro* did not significantly influence the mechanical properties of scaffolds, but that cell number was similar on all scaffolds. It is unclear whether the increased proliferation of cells on GraftJacket or the increased collagen expression by cells on Conexa would have translated to an increased tensile strength after 13 days. This would be an interesting point to explore in future work.

Results in this chapter contrast with a previous *in vitro* study by Shea *et al.* (342). Shea *et al.* (342) did show some similarities to this study, finding significantly increased collagen I and collagen III mRNA expression on Conexa. However, Shea *et al.* (342) also found significantly increased collagen I mRNA expression on GraftJacket and increased collagen III mRNA expression on GraftJacket and Permacol compared to four other commercially available scaffolds. These differences may be due to different sampling time points or the use of different cell types; we utilised cells from rotator cuff tear patients, while Shea *et al.* used tenocytes derived from healthy tendons that were discarded during orthopaedic procedures (342). Indeed, underlying changes in the tissue precede rotator cuff tears (122), therefore the phenotype of cells within diseased tissues is likely to be different. The differential

response between healthy and patient-derived cells may have important implications when evaluating the cellular response to biomaterials *in vitro*.

Marked differences in cell morphology were observed on the different scaffolds. After 1 day, cells on all scaffolds appeared rounded in comparison to tissue culture plastic (TCP), where cells were flattened. In contrast, after 13 days cell morphology differed markedly between the scaffold types tested. Cells on synthetic scaffolds aligned by following the orientation of the fibres, while cells on biosynthetic or dermal scaffolds were more isotropic and extended in numerous different directions, similar to TCP. Another interesting observation is the difference in cell-spread area between the synthetic and biological scaffolds. Cells on synthetic scaffolds are relatively more spread, with a greater number of actin stress fibres, which is also observed on TCP. In contrast, cells on biological scaffolds spread less. A link between increased cell spreading and increased surface modulus has been observed (234, 390) and correlates well with the surface increased elastic modulus values on the synthetic compared to biological scaffolds in this study, as determined in chapter 2 (figure 2-4B).

Distinct differences in cell morphology between the dermal scaffolds were observed; cells on human dermal scaffolds appeared smooth and interconnected, whereas cells on porcine dermal scaffolds appeared dendritic and punctate. Previous work suggests that the morphology of fibroblast-like cells transition from dendritic to smooth when the substrate they are on is sufficiently stiff (391). However, this does not fit with our previous work,

because the surface of GraftJacket is significantly more elastic (less stiff) than X-Repair, LARS ligament, Poly-Tape, and Conexa. Again, it seems that biochemical cues, such as native growth factors, are overriding the possible effects of biophysical cues, such as surface roughness and surface stiffness.

The results from this study suggest that the addition of collagen to BioFiber to make BioFiber-CM did not significantly affect cell attachment, proliferation, or gene expression *in vitro*. Conflicting results exist in the literature, with some studies reporting improved cellular response with collagen-coated biomaterials (392, 393) and some studies reporting no differences (394, 395). In tendon, tenocytes are organised into rows of connected cells within a pericellular matrix (396) and their behaviour is greatly influenced by their connectivity with other cells (397). Examination of cell morphology after 13 days revealed a sparse distribution of cells which did not form the connected networks like those seen on BioFiber (figure 3-6L). This is rather surprising and merits further investigation, especially within an *in vivo* system. It may simply be that the deposited collagen did not form a pattern conducive to the formation of aligned cell arrays. Alternatively, there may be other variables in the process of coating, such as denaturation of collagen, which may have affected the response of the cells to the surface.

There are several limitations of our work, which must be considered. Firstly, we used cells from a small number of patients (n=3) and the response of cells from other patients may differ. Secondly, we only studied one cell type, in isolation, and it is likely that the interaction between native tenocytes

and other cell types, such as macrophages, determine the patient's response to a scaffold. Finally, the study was performed in static tissue culture and was therefore unable to replicate the complex mechanical loading profile that the scaffolds would experience *in vivo*. These factors must be considered and as such, the relevance of our *in vitro* findings to clinical performance is limited.

The main strengths of this work include comparing a large number of scaffolds, which enable accurate comparisons between the various scaffolds tested. In addition, this study has a unique strength of utilising patient-derived cells from diseased tissue. This is of particular value because of the absence of good rotator cuff animal models.

3.7 Conclusions

Overall, this study suggests that synthetic scaffolds with large effective surface areas promoted increased cell attachment, minimally processed human dermis promote cellular proliferation, and non-cross-linked porcine dermis promotes the expression of collagen types I and III by patient-derived tenocytes. By revealing these potential advantages and disadvantages of each scaffold type, our data may help determine which scaffold is most appropriate for augmenting surgical rotator cuff repairs.

Future work in the field should use cells from diseased tissue to better understand the native biological mechanisms involved in rotator cuff repair and how scaffolds can be used to enhance these processes. Also, well-designed randomised control clinical trials comparing different scaffolds will help surgeons determine the most appropriate type of scaffold for clinical use. The influence that industrial processing techniques and collagen coating has on the cellular response to biomaterials warrants further investigation. In addition, how other types of cell present at the site of implantation, such as macrophages, interact with scaffolds are important to understand how scaffolds may perform *in vivo*.

Chapter 4 : The response of macrophages to rotator cuff scaffolds

4.1 Abstract

4.1.1 Background

Many scaffolds are available to augment rotator cuff repair during surgery. Following implantation, scaffolds induce a foreign body reaction (FBR), which can influence the quality of the repair. Macrophages are a key cell type contributing to the FBR, yet little is known about the response of human macrophages to commonly used allograft, cross-linked xenograft, non-cross-linked xenograft, and synthetic scaffolds. Furthermore, it is unclear how the response of macrophages to scaffolds influences the activity of human rotator cuff tenocytes.

4.1.2 Materials and methods

This study used an *in vitro* model to evaluate the response of human-derived macrophages (n=6) to four commercially available scaffolds. The scaffolds tested were a human dermis (GraftJacket), a cross-linked porcine dermis (Permacol), a non-cross-linked porcine dermis (Conexa), and a polyethylene terephthalate scaffold (LARS ligament). Expression of inflammatory genes (mRNA) and cytokines (protein) was evaluated 24 hours and 10 days after cell seeding. Supernatant from these studies was added to cultures of human rotator cuff tenocytes to investigate the effect that released cytokines (from macrophages) have on tenocyte proliferation and gene expression. The ability of the scaffolds to promote the formation of foreign body giant cells (FBGCs)

was also investigated by adding interleukin 4 (IL-4) to growth medium for 10 days.

4.1.3 Results

The results from this study demonstrated increased levels of cytokines including IL-1 β , IL-6, IL-10, and GM-CSF by macrophages on Conexa after 24 hours. While cytokine levels reduced for all scaffolds over 10 days, increased cytokine expression of IL-8, IL-10, and GM-CSF was observed in macrophages on Conexa. Supernatant from macrophages that had been seeded on Conexa caused tenocytes to decrease gene expression of *COL6A1* and increase expression of *MMP1* and *MMP3*. More FBGCs formed on Permacol after 10 days compared to all other scaffolds. Macrophages on GraftJacket and LARS ligament were associated with low cytokine expression and few FBGCs.

4.1.4 Conclusions

This study showed that human-derived macrophages express high levels of inflammatory cytokines when seeded on a non-cross-linked porcine scaffold (Conexa). We also demonstrate that the cytokines released prompted a catabolic response in rotator cuff tenocytes. Although cytokine release decreased over time in macrophages on all scaffolds, scaffolds that induced the greatest cytokine expression after 24 hours also promoted higher cytokine expression after 10 days. This suggests that the macrophage response towards a scaffold is maintained over time. In addition, FBGCs were more readily formed on a cross-linked porcine scaffold.

In conclusion, human-derived macrophages generate a more pro-inflammatory response towards non-cross-linked and cross-linked xenografts, compared to allograft and synthetic scaffolds *in vitro*. The data presented in this chapter could be used to better understand how macrophages interact with scaffolds *in vivo*.

4.2 Introduction

Scaffolds are implantable biomaterials used in the surgical repair of rotator cuff tendons. Following surgical implantation into the shoulder, a patient's immune system will mount a response against the scaffold, known as the foreign body reaction (FBR). The FBR is a sequence of events, beginning initially with the adsorption of proteins from the serum and synovial fluid to the surface of a scaffold to form the provisional matrix (398). This is followed by the invasion of inflammatory cells such as neutrophils, monocytes/macrophages, and mast cells (353). If the FBR does not spontaneously resolve, the FBR can develop into a chronic inflammatory response, which is characterised by the fusion of macrophages to form foreign body giant cells (FBGCs)(399). FBGCs develop when macrophages are unable to individually phagocytose foreign objects, which can include certain types of scaffold.

While many cell types are involved in the FBR, macrophages and monocytes, from which macrophages derive, are a fundamental component (353, 400-405). Macrophages regulate the FBR by responding to environmental cues and secreting a range of cytokines (353, 402, 404, 405) that affect cells in the surrounding milieu. The tenocyte is the most abundant cell type in the rotator cuff and would therefore be the tissue cell that was most affected by any proximal macrophage response towards a scaffold.

Previous work has demonstrated that macrophage-secreted cytokines can influence the proliferation and collagen expression of fibroblasts (406).

Macrophages can also respond to scaffolds by differentiating into specialised sub-types (407, 408) and other specialised cells, such as FBGCs (409, 410). Therefore, it is clear the way that macrophages respond to implanted scaffolds can significantly impact the healing process (353, 404, 411-413).

Macrophages can secrete a range of inflammatory cytokines, depending upon the signals that they receive from the environment. A macrophage can be stimulated to secrete pro-inflammatory cytokines in response to a range of stimuli including pathogen associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS)(414). Pro-inflammatory cytokines include interleukin-1 beta (IL-1 β), IL-6, IL-8, and tumour necrosis factor-alpha (TNF- α).

In contrast, macrophages can be stimulated to release anti-inflammatory cytokines by exposure to IL-4, IL-10, and IL-13. Anti-inflammatory cytokines include interleukin 1 receptor antagonist (IL-1RA) and IL-10. While this rigid categorisation of cytokines can be convenient, it fails to account for the fact that many cytokines have both pro-inflammatory and anti-inflammatory effects (405, 408, 415). It is also noteworthy that not all types of inflammation are deleterious. Even though increased inflammation has been associated with rotator cuff tears (57, 121), inflammation is also a necessary component of the tendon healing process (43, 416).

Previous studies have demonstrated that implantable scaffolds can influence the type of cytokines that macrophages secrete (261, 417, 418) depending on scaffold characteristics such as surface chemistry (411, 419),

stiffness (420), topography (47), and hydrophilicity (411). Several previous *in vitro* studies have explored the cytokine profiles that macrophages produce in response to surgical scaffolds. Orenstein *et al.* (417) evaluated the expression of IL-1 β , IL-6, IL-8, and vascular endothelial growth factor (VEGF) by macrophages in response to four porcine scaffolds (CollaMend, Permacol, Strattice, and Surgisis). The authors reported increased expression of all cytokines on the cross-linked scaffolds compared to the non-cross-linked scaffolds (417).

Orenstein *et al.* (261) also studied the amount of IL-1 β macrophages released when seeded onto three non-cross-linked human dermal scaffolds. The study demonstrated differential expression of IL-1 β , suggesting that different processing techniques used to create each scaffold likely influenced the macrophage response (261). Grotenhuis *et al.* (418) also analysed cytokine expression after seeding human mononuclear cells on two porcine scaffolds and two synthetic scaffolds *in vitro*. The study showed that the synthetic scaffolds promoted greater expression of pro-inflammatory cytokines (418).

Concerns have also been raised regarding remnant DNA in biological scaffolds because of its association with an increased inflammatory response (421). DNA is often used as a surrogate marker for measuring the number of residual cells and it is unlikely that any biological scaffolds will be completely decellularised (184). Several studies have demonstrated that many biological scaffolds retain some native DNA (195, 422), but the precise implications of this remains unclear.

Allograft, xenograft, and synthetic scaffolds being among the most popular types of scaffold used for rotator cuff repair and macrophages are important in the response to implanted scaffolds. However, no previous study has directly compared the macrophage response to these scaffold types. Additionally, few *in vitro* studies have assessed the ability of commercial scaffolds to promote the formation of FBGCs and a limited number have evaluated the effect that macrophage-secreted cytokines have upon human rotator cuff tenocytes.

4.3 Aims and Hypotheses

Therefore, the first aim of the study was to evaluate and compare the *in vitro* response of human macrophages to commercially available allograft, xenograft, and synthetic scaffolds. One allograft dermal scaffold (GraftJacket), one cross-linked porcine dermal scaffold (Permacol), one non-cross-linked porcine dermal scaffold (Conexa), and one synthetic scaffold (LARS ligament) were investigated *in vitro*. These four scaffolds were chosen to represent a cross-section of commercially available scaffolds. The second aim of the study was to determine whether macrophage-secreted cytokines released in response to scaffolds could influence the proliferation and gene expression of rotator cuff tenocytes.

Based upon previous studies, it was hypothesised that the synthetic scaffold would promote a greater pro-inflammatory response than the allograft or xenograft scaffolds. It was also hypothesised that cytokines released from macrophages on the synthetic scaffold would significantly reduce tenocyte proliferation and collagen expression.

4.4 Materials and Methods

Following the monocyte isolation step, all subsequent parts of the experiment will describe the cells as macrophages for simplicity.

4.4.1 Scaffold selection and preparation

Four commercially available scaffolds were selected: one human dermal scaffold (GraftJacket), one cross-linked porcine dermal scaffold (Permacol), one non-cross-linked porcine dermal scaffold (Conexa), and one synthetic scaffold made from polyethylene terephthalate (LARS ligament). All scaffolds were prepared according to manufacturers' instructions. Scaffolds were cut into circular discs of 11mm diameter using a custom-made biopsy punch, such that each scaffold piece filled one well of a 48-well plate. Prior to cell seeding, scaffolds were incubated for 24 hours in 1mL of monocyte medium (X-VIVO hematopoietic media (Lonza) supplemented with 10% heat-inactivated human serum (Sigma).

4.4.2 Monocyte isolation and seeding

Peripheral blood was collected, with informed consent, from six healthy volunteers from. Cones of blood were purchased and subsequently used for mononuclear cell isolation. Mononuclear cells were isolated from peripheral blood using a previously established protocol.

Briefly, buffy coats of blood were diluted in phosphate-buffered saline (PBS, Lonza) and cells were centrifuged using a Ficoll gradient for 25mins at 1100g at room temperature. The white intermediate ring of peripheral blood mononuclear cells (PBMCs) was removed and rinsed in PBS. The isolated

PBMCs were centrifuged again for 10mins at 1100g at room temperature. Supernatant was removed and cells were re-suspended in PBS before centrifugation for a further 10mins at 930rpm. The cell pellet was then re-suspended in 30ml of RPMI media (Sigma Aldrich) and 5ml of cells were layered onto 5ml of Percoll gradient (46% in RPMI media) (423). Cells were centrifuged for 25mins at 2000rpm. The white intermediary ring, consisting of monocytes, was isolated and washed in PBS. Monocytes were centrifuged for 25mins at 2000rpm, and the resulting cell pellet was washed with PBS. Monocytes were centrifuged for 10mins at 1000rpm, and the resulting cell pellet was washed with PBS. Monocytes were centrifuged for 10mins at 600rpm, and the resulting cell pellet was re-suspended in monocyte medium. Monocyte selection was further enriched using CD14 magnetic MicroBeads (Miltenyi Biotec).

Cells from each donor were seeded at 500,000 per well onto each of the four scaffolds in a 48-well plate. Positive controls (TCP+LPS) were 500,000 monocytes on tissue culture plastic in a 48-well plate supplemented with 10ng/ml LPS (Sigma Aldrich). Negative control was 500,000 monocytes on tissue culture plastic (TCP) alone. For the 10-day culture, fresh medium was replenished on day 4 and day 7. A 10 day time point was chosen because it is a long enough *in vitro* culture time to promote differentiation of monocytes to macrophages (424).

4.4.3 DNA quantification

DNA was quantified 24 hours and 10 days after cell seeding onto 11mm discs (n=6 per time point, one per patient), to approximate the number of cells present on each scaffold at each time point.

Scaffolds were rinsed twice in PBS (Sigma-Aldrich) then suspended in 350µL of 0.1% Triton X-100 (Sigma). Scaffolds were freeze-thawed three times and sonicated twice for 10 s using an ultrasonic cell disruptor (Misonix, NY, USA). DNA quantification was performed using the Quant-iT PicoGreen assay (Invitrogen, UK) according to manufacturer's instruction. Briefly, 50µl of each sample was added to 50µl of 1X Tris-EDTA buffer and 100µl of the PicoGreen reagent to make a 200µl final reaction volume in an opaque 96-well plate (Greiner). Samples were incubated for 5 minutes in the dark, and then fluorescence was measured at 485nm excitation and 520nm emission using a FLUOstar Omega micro-plate reader (BMG Labtech). No-cell controls (n=3 per scaffold) were used to quantify any native DNA, which was accounted for by subtracted it from experimental readings.

4.4.4 Macrophage gene expression analysis

On day 1 and day 10, cell-seeded scaffolds were fixed in 350µl of TRIzol (Invitrogen) and mRNA was extracted using the Direct-zol RNA miniprep kit (Zymo Research). The mRNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Synthesised cDNA was diluted to 5ng/µl and 1µl was used in a 10µl RT-qPCR reaction with Fast SYBR Master Mix (Applied Biosystems) using a ViiA7 real-time

PCR machine (Applied Biosystems). Beta-actin (*ACTB*) and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were used as endogenous reference genes, and relative mRNA expression was calculated using the comparative CT method (351). Primers for *GAPDH*, *CSF2*, *IL1RN*, and *IL4* (Qiagen) were used in addition to primers for *ACTB*, *TNF*, *IL1B*, *IL8*, *IL12B*, *TGFB1*, *IL6*, and *IL10* (sequences in Appendix B).

4.4.5 Macrophage cytokine analysis

On day 1 and day 10, medium was removed from scaffolds and control wells and placed in 1.5ml eppendorf tubes. Medium was centrifuged for 2 minutes at 12,000rpm and the resulting supernatant was removed and placed into clean 1.5ml eppendorf tubes, then frozen at -80oC prior to cytokine analysis. Secreted cytokines were quantified using a human pro-inflammatory 9-plex tissue culture kit (Meso scale discovery) according to manufacturer's instructions. Cytokines analysed were TNF- α , IL-1 β , IL-8, IL-12p70, GM-CSF, IFN- γ , IL-2, IL-6, and IL-10.

4.4.6 Tenocyte isolation and tissue culture

Tenocytes were isolated from supraspinatus tendon biopsies from six patients (three male, three female, aged 44-79) with tendinopathy undergoing rotator cuff repair or subacromial decompression. Tissue samples were collected with informed donor consent under the ethics from the Oxford Musculoskeletal Biobank (09/H0606/11). Cells were isolated using a previously established protocol (316). Briefly, tissue biopsies were cut into 2mm squares and cultured in 6-well plates (Corning) in DMEM-F12 (Lonza) supplemented with

50% fetal bovine serum (Labtech, UK) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific). Media was replenished every 3 days. Once cells reached confluence, they were transferred to 10cm dishes (Greiner) and expanded to passage 3 prior to use. Tenocytes from six patients were seeded separately at 25,000 per well in 24-well plates (Corning) in supplemented DMEM-F12 media (Lonza).

4.4.7 Tenocyte proliferation and gene expression analysis

One day after tenocyte cell seeding, 1ml of 5% AlamarBlue solution (Invitrogen) in DMEM-F12 medium supplemented with 10% fetal bovine serum (Labtech, UK) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific) was added to each well for 2 hours. Next, 100µl of the AlamarBlue solution was added to an opaque 96-well plate (Greiner) and fluorescence was measured at 485nm excitation and 520nm emission using a FLUOstar Omega micro-plate reader (BMG Labtech). This was used as a baseline reading for cell number.

To assess the effect of monocyte-secreted cytokines on tenocyte behaviour, 250µl of supernatant from each of the monocyte experiments (day 1 and day 10) was mixed with 250µl of fresh monocyte medium and added to each well, containing tenocytes (supernatant-enriched medium). A well containing just 250µl of supplemented X-VIVO was used as a negative control. Tenocytes were incubated in supernatant-enriched medium for 48 hours, after which time, cell number was determined again using the AlamarBlue assay. Samples were then fixed, mRNA was extracted, cDNA

created and RT-qPCR performed as outlined above. Primers for *GAPDH*, *TBP*, *COL1A1*, *COL3A1*, *COL6A1*, *MMP1*, *MMP3*, *TGFB1*, *TNC*, and *DCN* were used (Qiagen).

4.4.8 Foreign body giant cell formation

Isolated monocytes were seeded onto each scaffold (n=6 patients) at 500,000 monocytes per well in a 48-well-plate. Positive controls (TCP+IL-4) were monocytes cultured in monocyte medium supplemented with 10ng/ml IL-4. Negative controls (TCP) were monocytes cultured in monocyte medium alone. On day 4 and day 7, monocyte medium supplemented with 10ng/ml IL-4 was added to all scaffolds and positive controls to encourage foreign-body giant cell formation (FBGC), as previously described (425). After 10 days, scaffolds were rinsed twice in pre-warmed PBS and fixed in 10% formalin for 2 hours. Scaffolds were again rinsed twice in PBS. 1ml of 0.1% Triton-X was added to each sample for 10 minutes, and samples were then rinsed twice in PBS. Then 500 µl of 100nM solution of acti-stain 488 phalloidin (Cytoskeleton Inc.) and 4', 6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific) in PBS was added to each sample. Samples were incubated for three hours in the dark at 4°C, rinsed twice in PBS and images were captured at 10X (for cell counting) and 20X (for qualitative analysis) magnification using a Zeiss LSM710 confocal microscope (Zeiss).

Three images per scaffold per patient of random areas were acquired at 10X (n=6 patients). FBGCs were identified by the fusion of five or more cells and were counted. This was performed using the CellProfiler (426, 427) image

analysis software. The mean number of FBGCs for each patient was calculated from the total number of FBGCs in the three images acquired. The mean size (mean number of cells per FBGC) was also measured.

4.4.9 Statistical analysis

A Kolomogorov-Smirnov tested was used to determine normality for each experiment. If normality was determined, a one-way ANOVA was performed with a parametric Tukey *post-hoc* test to compare all scaffolds tested. All statistical analyses were performed using GraphPad PRISM (GraphPad Software Inc.). Statistical significance was determined at $*\leq 0.05$, $**\leq 0.01$, and $***\leq 0.001$ and 95% confidence intervals (CIs) of minimum difference were reported.

4.5 Results

4.5.1 Native DNA content of scaffolds and the effect of scaffolds on monocyte cell attachment and proliferation

Native scaffold DNA was compared between the four scaffolds—no positive or negative controls were used. Native scaffold DNA was significantly higher on GraftJacket compared to every other scaffold ($p \leq 0.001$, 95% CI: 633, 767) (figure 4-1A). Permacol had significantly more native DNA than Conexa ($p \leq 0.01$, 95% CI: 39, 173) and LARS ligament ($p \leq 0.001$, 95% CI: 76, 210) (figure 4-1A). After 24 hours following cell seeding there was no difference in monocyte attachment as measured by DNA content (figure 4-1B). After 10 days, more cells were attached to GraftJacket ($p \leq 0.05$, 95% CI: 67, 2307) than every other scaffold (figure 4-1C). For figures 4-1B and 4-1C, positive controls were tissue culture plastic (TCP) with lipopolysaccharide (LPS) and negative controls were just TCP alone.

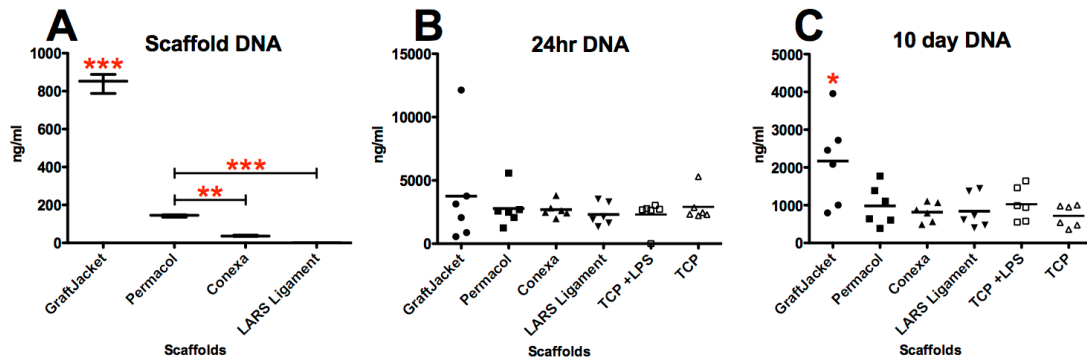


Figure 4-1. Native scaffold DNA content and DNA content 24 hours and 10 days following cell seeding.

Native scaffold DNA content (A), DNA content 24 hours after cell seeding (B), and 10 days after cell seeding (C). Red asterisks denote significant differences between scaffolds. In figure (C), the red asterisk denotes that GraftJacket had significantly more DNA (cells) attached compared to every other scaffold ($p \leq 0.05$). Figure A is a boxplot. Figures B and C plot the individual values and the horizontal line represents the median.

4.5.2 Inflammatory gene expression profile of scaffold-cultured monocytes

After 24 hours, gene expression of *TNF* ($p \leq 0.001$, 95% CI: 0.10, 0.25) (figure 4-2A), *IL12B* ($p \leq 0.001$, 95% CI: 0.08, 0.14) (figure 4-2E), and *TGFB1* ($p \leq 0.001$, 95% CI: 0.09, 0.14) (figure 4-2F), was significantly higher in cells on GraftJacket compared to all other scaffolds. LARS ligament was the most potent inducer of *IL1RN* ($p \leq 0.01$, 95% CI: 0.036, 0.22) (figure 4-2G) after 24 hours, in addition to up-regulating expression of *TGFβ* when compared to Conexa ($p \leq 0.05$, 95% CI: 0.00061, 0.054) (figure 4-2F).

After 10 days, gene expression of *TNF* ($p \leq 0.01$, 95% CI: 0.18, 1.44) (figure 4-2A), *IL1B* ($p \leq 0.05$, 95% CI: 0.019, 0.39) (figure 4-2B), *IL6* ($p \leq 0.01$, 95%

CI: 0.0046, 0.025) (figure 4-2C), *IL8* ($p \leq 0.001$, 95% CI: 0.088, 0.34) (figure 4-2D), *TGFB* ($p \leq 0.01$, 95% CI: 0.085, 0.67) (figure 4-2F), and *IL4* ($p \leq 0.05$, 0.00052, 0.016) (figure 4-2H) mRNA were significantly increased in cells on GraftJacket compared to all other scaffolds.

Gene expression of *CSF2*, *IL10*, and *IFN* was also evaluated but no significant differences between scaffolds were observed at either time point.

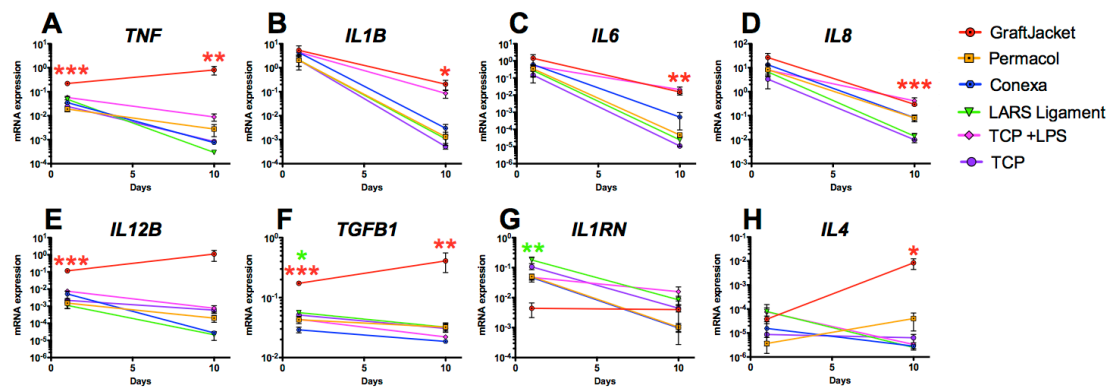


Figure 4-2. Quantification of macrophage mRNA on scaffolds over 10 days.

mRNA expression was calculated relative to GAPDH. *TNF* (A), *IL1B* (B), *IL6* (C), *IL8* (D), *IL12B* (E), *TGFB1* (F), *IL1RN* (G), and *IL4* (H). Asterisks are color-coded based in the scaffold key (right of figure) and denote significant differences between each scaffold and every other scaffold at the same time point. Figures are boxplots connected by straight lines.

4.5.3 Effect of scaffold type on secretion of inflammatory cytokines by macrophages

Approximately 24 hours after macrophage seeding, secretion of IL-1 β ($p \leq 0.05$, 95% CI: 0.011, 0.29) (figure 4-3B), GM-CSF ($p \leq 0.01$, 95% CI: 0.067, 0.34) (figure 4-3C), IL-6 ($p \leq 0.01$, 95% CI: 0.69, 4.68) (figure 4-3D), and IL-10 ($p \leq 0.01$, 95% CI: 0.018, 0.091) (figure 4-3F) was significantly elevated in cells on Conexa compared to all other scaffolds. TNF- α was also higher in cells on Conexa ($p \leq 0.05$, 95% CI: 0.23, 1.44) compared to the other scaffolds tested (figure 4-3A). No other differences were observed after 24 hours.

After 10 days, cytokine secretion of IL-10 ($p \leq 0.01$, 95% CI: 0.0015, 0.0080) (figure 4-3F) was significantly higher in cells on Conexa compared to all other scaffolds. Also, secretion of GM-CSF was higher on Conexa compared to GraftJacket ($p \leq 0.05$, 95% CI: 0.0086, 0.081) and Permacol ($p \leq 0.05$, 95% CI: 0.0029, 0.075) (figure 4-3C). IL-8 secretion was higher on Conexa compared to GraftJacket ($p \leq 0.001$, 95% CI: 4.97, 20.33) and Permacol ($p \leq 0.01$, 95% CI: 2.37, 17.74) (figure 4-3E).

Cytokine secretion of IL-12p70, IFN- γ , and IL-2 were also evaluated but no significant differences between scaffolds were observed at either time point.

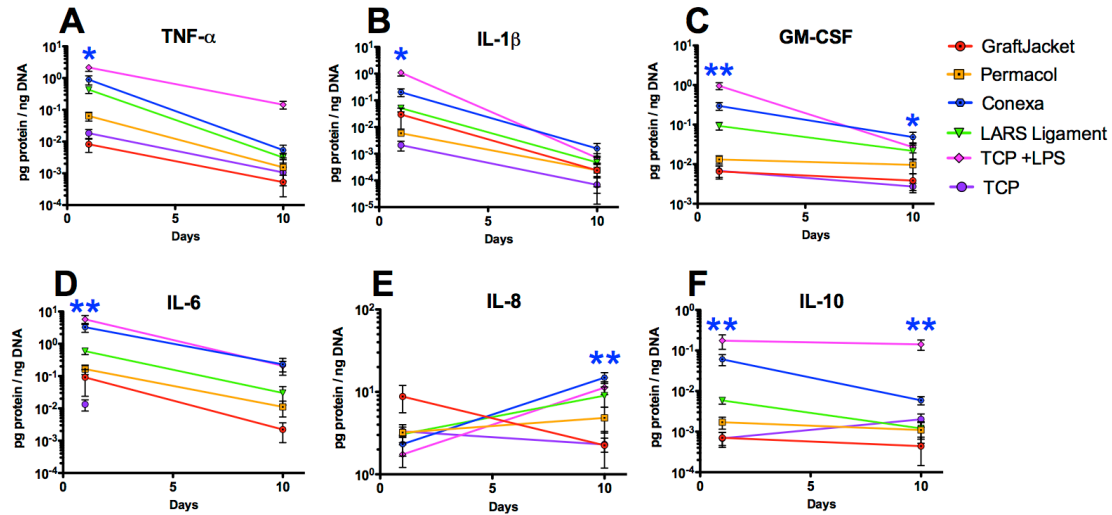


Figure 4-3. Quantification of inflammatory cytokines secreted by macrophages on scaffolds over 10 days.

Cytokine expression was standardised to DNA content (after subtracting any native DNA content). Figures are boxplots connected by straight lines. TNF- α (A), IL-1 β (B), GM-CSF (C), IL-6 (D), IL-8 (E), and IL-10 (F). Asterisks are color-coded based in the scaffold key (right of figure) and denote significant differences between that each scaffold and every other scaffold at the same time point except:

- 1 day reading of TNF- α on Conexa is only significantly different compared to GraftJacket and Permacol.
- 10 day reading of GM-CSF on Conexa only significantly different compared to GraftJacket and Permacol.
- 10 day reading of IL-8 on Conexa only significantly different compared to GraftJacket and Permacol.

4.5.4 Effect of monocyte-derived supernatant-enriched medium on tenocyte proliferation and gene expression

The supernatant from the macrophage-scaffold experiments was removed after 24 hours. This was added to tenocytes (alone) for 48 hours in a separate culture dish. The supernatant from different scaffolds (and macrophages) did not significantly affect tenocyte proliferation. However, tenocytes exposed to Conexa's monocyte-derived supernatant demonstrated a reduced expression of *COL6A1*, when compared to GraftJacket ($p \leq 0.01$, 95% CI: 0.013, 0.077) and Permacol ($p \leq 0.05$, 95% CI: 0.0031, 0.67) (figure 4-4A). In addition, Conexa's supernatant significantly increased tenocyte expression of *MMP-3* ($p \leq 0.01$, 95% CI: 0.51, 2.64) (figure 4-4E) after 48 hours. Conexa's supernatant significantly increased tenocyte expression of *MMP-1* relative to Permacol ($p \leq 0.05$, 95% CI: 0.016, 0.89) after 48 hours (figure 4-4D). GraftJacket's supernatant significantly increased *TGF- β* expression relative to LARS ligament ($p \leq 0.05$, 95% CI: 0.0012, 0.027) (figure 4-4B).

After 48 hours exposure to 10-day macrophage supernatant, no differences in gene expression or proliferation were seen in rotator cuff tenocytes (figure 4-5).

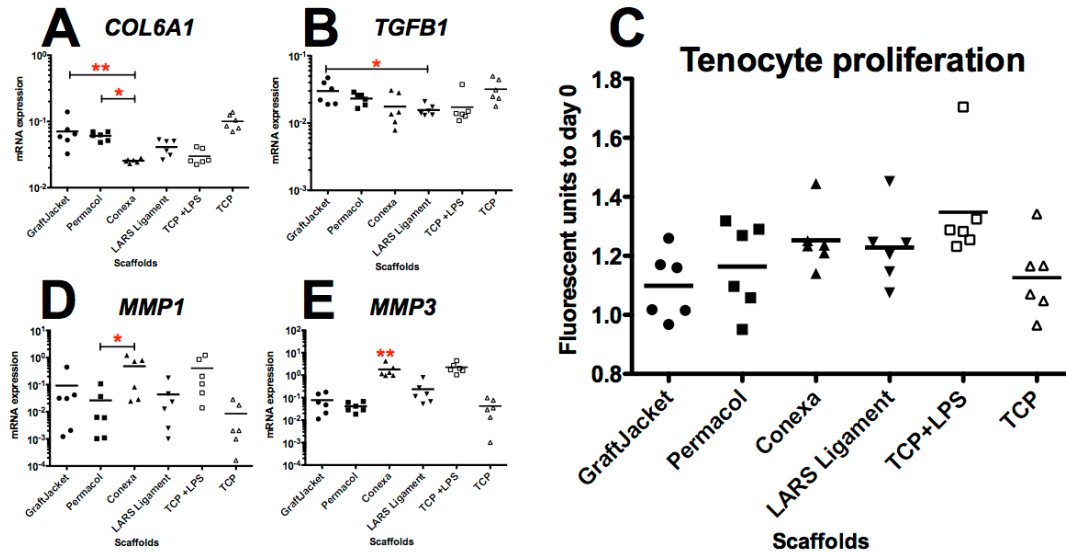


Figure 4-4. Quantification of tenocyte gene expression and proliferation after 48 hours exposure to supernatant-enriched medium from the 24 hours macrophage-scaffold experiment.

COL6A1 (A), *TGFB1* (B), *MMP1* (D), and *MMP3* (E). Tenocyte proliferation over 48 hours (C). Red asterisks designate significant differences between scaffolds. Figures plot the individual values and the horizontal line represents the median.

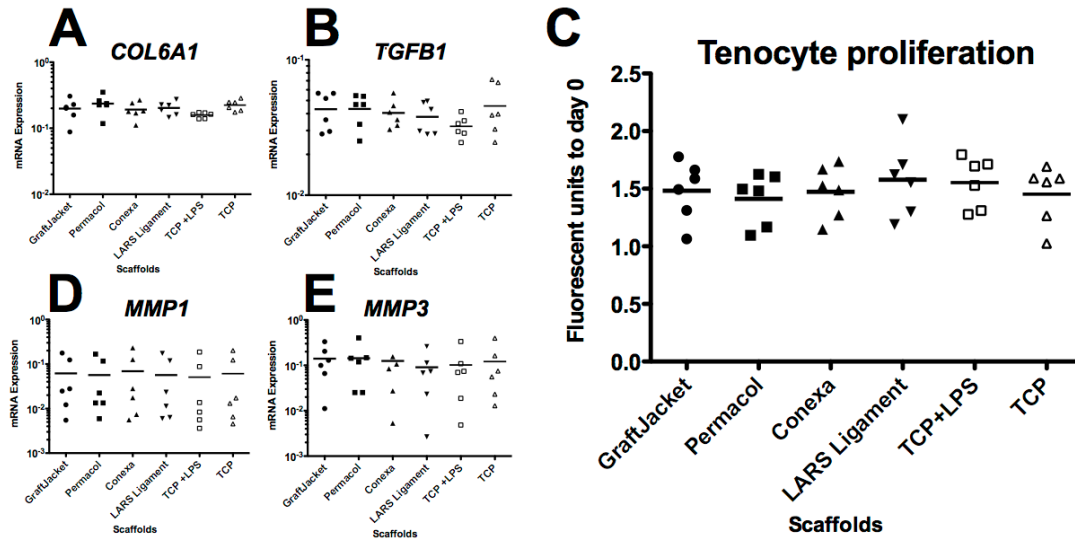


Figure 4-5. Quantification of tenocyte gene expression and proliferation after 48 hours exposure to supernatant-enriched medium from the 10-day macrophage-scaffold experiment.

COL6A1 (A), *TGFB1* (B), *MMP1* (D), and *MMP3* (E). Tenocyte proliferation over 48 hours (C). Figures plot the individual values and the horizontal line represents the median.

4.5.5 Effect of scaffold type on FBGC formation

After 10 days, more FBGCs formed on Permacol when compared to GraftJacket ($p \leq 0.05$, 95% CI: 4, 84) and LARS ligament ($p \leq 0.05$, 95% CI: 0.41, 80.25) (figure 4-6G). No significant differences in numbers of FBGCs were observed between Permacol and Conexa (figure 4-6G). There was no difference in the average size of FBGCs identified on each scaffold (figure 4-6H).

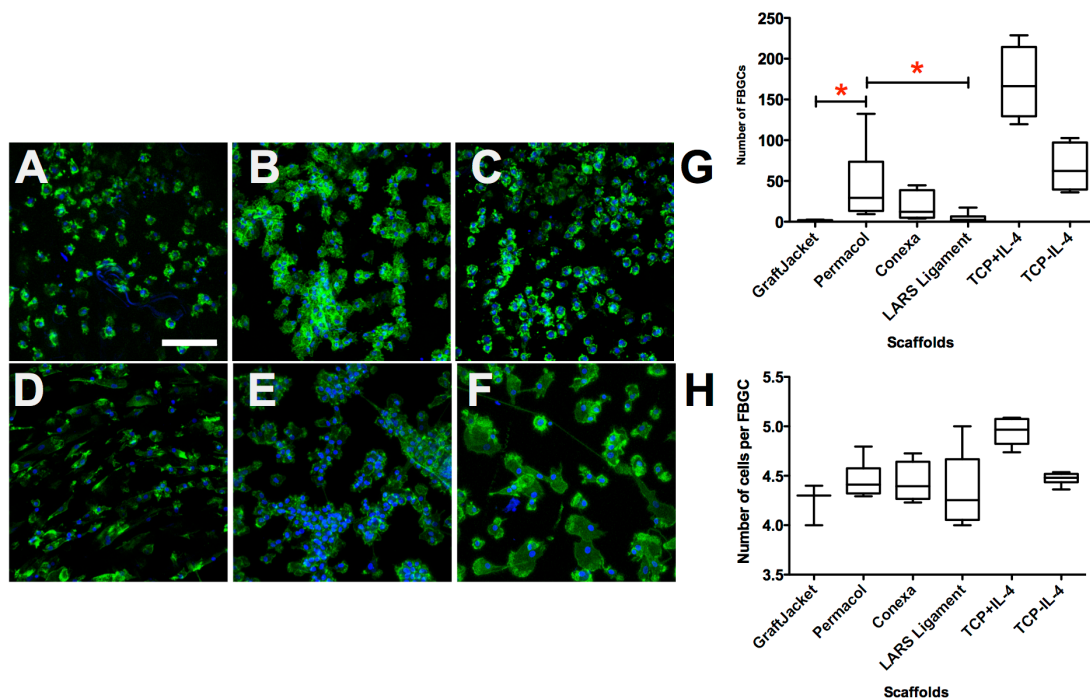


Figure 4-6. Foreign body giant cell (FBGC) formation on scaffolds after 10 days.

GraftJacket (A), Permacol (B), Conexa (C), LARS ligament (D), TCP + IL-4 (E), and TCP (F). The number of FBGCs on scaffolds (G) and the average number of cells per FBGC (H). Images taken at 20X. Scale bar= 100 μ m. Figures G and H represent boxplots. In figure 4-6H, GraftJacket only represents maximum, minimum, and medium because only 3 FBGCs were found.

4.6 Discussion

Numerous scaffolds are commercially available to augment the surgical repair of rotator cuff tendons. Following implantation, scaffolds experience a sequence of events that collectively form the foreign body reaction (FBR). Macrophages are a key immune cell type that modulates the FBR towards an implanted scaffold (353, 401, 404, 428). In this *in vitro* study, we evaluated the way that human macrophages respond to four commercially available scaffolds including a human allograft (GraftJacket), a non-cross-linked xenograft (Conexa), a cross-linked xenograft (Permacol) and synthetic scaffold (LARS ligament). We found that the non-cross-linked porcine dermal scaffold (Conexa) promoted the greatest release of pro-inflammatory cytokines after 24 hours and 10 days. The release of these cytokines prompted rotator cuff tenocytes to upregulate MMP and downregulate COL6A1 gene expression. The formation of FBGCs was greatest on the cross-linked porcine dermal scaffold (Permacol) after 10 days.

Attachment is a vital part of the macrophage response to a scaffold (353) and is mediated by receptors, such as integrins (429), that bind to ligands in adsorbed or surface-derived proteins. Cell attachment to scaffolds is influenced by a variety of factors including scaffold hydrophobicity (430), roughness (431), material modulus (420, 432), topography (433, 434), and surface chemistry (419). However, we found no difference in macrophage attachment between the scaffolds tested. This contrasts with Irwin *et al.* (432) who found that macrophages preferentially attach to surfaces with increased stiffness.

Work from chapter 2 (figure 2-4B) shows that LARS ligament and Conexa have higher surface stiffness compared to GraftJacket and Permacol. The results of our work align with those of Blakney *et al.* (420) who found no difference in macrophage attachment to hydrogels with different surface stiffness. Increased surface roughness has also been associated with increased macrophage attachment (435, 436), but work has also shown that surface roughness does not influence macrophage attachment (437). While the surface of GraftJacket, Permacol, and Conexa are rougher than LARS ligament (figure 2-6B), we found that surface roughness did not correlate with macrophage attachment.

Following attachment to the scaffold surface, monocytes differentiate into macrophages, secreting cytokines based on cues that cells receive from the scaffold and surrounding cells (399). After 24 hours, gene expression of *TNF*, *IL12B*, and *TGFB1* were significantly higher on GraftJacket compared to all other scaffolds. However, these gene expression changes were not reflected in cytokine levels in the supernatant. Discordance between mRNA and cytokine expression could be due to a variety of factors including variations in protein half-life (438), translational efficiency of mRNA transcripts (439), and post-transcriptional regulation (353, 440). Other factors may include the intra-cellular storage of translated protein, and the immediate consumption or adsorption of secreted cytokines.

A recent review highlighted the movement and storage of cytokines in intracellular vesicles by macrophages and other cell types (441). For example,

macrophages transport TNF- α in vesicles (442) and, after the addition of an inhibitor for the TNF- α converting enzyme (TACE), TNF- α accumulated at the cell-surface or remained stored in intracellular vesicles (443). Macrophages also transport IL-6 in vesicles, often together with TNF- α (444). Therefore, it is possible that something within the scaffolds affected the pathways involved in cytokine secretion or that the translated cytokines were just never secreted and therefore not detected. It is also possible that secreted cytokines were immediately consumed and several studies have demonstrated autocrine and paracrine effects of cytokines in macrophages (445, 446). However, the precise reason for this curious observation remains unclear and warrants further investigation.

After 24 hours, cytokine secretion of IL-1 β , IL-6, IL-10, and GM-CSF were significantly elevated in macrophages on Conexa, compared to every other scaffold. TNF- α expression was also increased in cells on Conexa compared to GraftJacket and Permacol. IL-10 is an anti-inflammatory cytokine (447), while GM-CSF, IL-6, TNF- α , and IL-1 β are pro-inflammatory cytokines (407). Although the capability of xenografts to promote human macrophages to express pro-inflammatory cytokines has been reported (417), this is the first study to show Conexa doing so. Human macrophages can respond to xenogeneic cell-surface antigens (448), such as galactose- α -1,3-galactose (α -gal)(449), which is present throughout the animal kingdom, but not in humans or old-world monkeys (450). Even though Conexa is enzymatically processed to reduce α -gal levels (451), human macrophages are also responsive to porcine cells deficient in the α -gal epitope (452). Given that

complete decellularisation of biologic scaffolds is unlikely (450), remnant xenogeneic cellular debris may have contributed to the release of pro-inflammatory cytokines that was observed on Conexa.

However, other explanations for these observations should be considered. Endogenous extracellular matrix proteins, such as hyaluronan (453), biglycan (454), and fibronectin (455) have been shown to alter macrophage behaviour and are all present in dermal tissue. Indeed, the ability for protein fragments from ECM scaffolds to influence cell behaviour has been observed (402, 456). The potential for human macrophages to respond differently to fragments from xenograft or allograft extracellular matrices has not been explored, but warrants further investigation.

Other surface properties may also have influenced the initial increase in inflammatory cytokines that we observed, but conflicting data exists. Blakney *et al.* (420) showed that more elastic (less stiff) hydrogels reduced the expression of IL-1 β , IL-6, and TNF- α *in vitro* and were associated with fewer macrophages *in vivo*. In contrast, Irwin *et al.* (432) found no difference in TNF- α expression on surfaces with different stiffness and found IL-8 expression to be attenuated by very soft or very stiff surfaces. Interestingly, we found that the surface of Conexa was significantly stiffer than GraftJacket and Permacol (figure 2-4B), which supports the study by Blakney *et al.* (420). However, this does not correlate with the reduced inflammatory cytokine secretion by macrophages in LARS ligament, which has a relatively stiff surface (figure 2-4B).

Increased roughness has also been correlated with the increased secretion of inflammatory cytokines by macrophages (436, 437), but previous work has also shown it to have no effect (435). The results of our study suggest that roughness has little effect on macrophage cytokine secretion, with GraftJacket, Permacol, and Conexa exhibiting similar surfaces roughness (figure 2-6B), but differences in the quantities of cytokines released.

DNA content has been implicated as a cause of immune response to biological scaffolds (421, 450), following the logic that it is a surrogate measure for remnant cellular debris. However, DNA does not always approximate cellular contamination (457) and the unexamined practice of using DNA content as a metric for immunogenicity has been questioned (458). Our results show increased pro-inflammatory cytokine release in macrophages on Conexa, despite it having the lowest DNA content between the three biological scaffolds tested.

Interestingly, Fadok *et al.* (446) showed that after ingesting apoptotic cells, human macrophages decreased secretion of IL-1 β , IL-8, IL-10, TNF- α , and GM-CSF. If native scaffold DNA does correlate with the number of remnant cells in our study, then the work by Fadok *et al.* (446) would help explain the cytokines results. Our study demonstrates an inverse correlation between native scaffold DNA and cytokine expression, which could be caused by the mechanisms postulated by Fadok *et al.* (446). However, further histological analysis of the native biological scaffolds is required to confirm that DNA is positively correlated with the number of remnant cells.

Even though Permacol, like Conexa, is derived from porcine dermis, we observed reduced expression of pro-inflammatory cytokines compared to Conexa. This may be partially explained by the fact that Permacol is cross-linked (459), while Conexa is not. Cross-linking reduces degradation rate (178, 460, 461), which may have slowed the release of macrophage-activating antigens. Also, cross-linking can mask antigenic epitopes and reduce activation of immune cells (450). A study by Grotenhuis *et al.* (418) demonstrated reduced levels of pro-inflammatory cytokines released by human macrophages on Permacol.

In addition, Umashankar *et al.* (462) found that macrophages on lightly cross-linked xenografts expressed lower levels of TNF- α and IL-1 β compared to non-cross linked bovine xenografts. In contrast, Orenstein *et al.* (417) reported increased expression of IL-1 β by human macrophages in response to Permacol compared to non-cross-linked porcine dermal scaffolds, with the authors suggesting that cross-linking was a major cause of this. However, concerns have been raised regarding the conclusions of this study (463), as the authors did not standardise cytokine expression to cell number. While cross-linking may contribute to the reduction in macrophage activation, it is likely that other scaffold-related factors such as scaffold source, also play a role.

Our study demonstrated that the allograft (GraftJacket) and the synthetic scaffold (LARS ligament) did not induce a significant increase in pro-inflammatory cytokine expression by macrophages. However, other human dermal scaffolds (261) and polyethylene terephthalate (418) have been

shown to induce pro-inflammatory cytokine expression. Our results are not necessarily at odds with these studies, just that any induction is less than that induced by GraftJacket and LARS ligament relative to Conexa.

After 10 days, gene expression of *TNF*, *IL1B*, *IL8*, *TGFB1*, *IL4*, and *IL6* was significantly higher in macrophages cultured on GraftJacket compared to other scaffolds. However, again, we did not see the same pattern at the protein level. After 10 days, secretion of IL-10 cytokine was higher on Conexa than all other scaffolds. IL-8 and GM-CSF secretion were higher on Conexa compared to GraftJacket and Permacol, and IL-2 was more highly expressed on Conexa compared to GraftJacket.

In general, cytokine expression decreased over 10 days, suggesting partial resolution of the initial inflammatory response. The only exception was IL-8 in cells on Conexa, which actually increased over 10 days. IL-8 has been implicated in angiogenesis (464) and is the main recruiting and activating molecule of neutrophils (465), which could indicate the maintenance of inflammation in macrophages on Conexa. Despite decreased cytokine expression over 10 days, macrophages on Conexa still released relatively more pro-inflammatory cytokines than those on the other scaffolds. This suggests that scaffolds-derived signals that promote the expression of pro-inflammatory cytokines are maintained over time. Identifying how these signals influence macrophage behaviour will be of value to the field, and our study provides a foundation upon which future work can build.

Macrophages secrete cytokines that influence the behaviour of other cell types in the surrounding milieu (466). Tenocytes are the major cell type within the rotator cuff tendons. These fibroblast-like cells regulate tissue turnover by expressing extracellular matrix proteins, such as collagens (467) and catabolic products such as matrix metalloproteinases (MMPs)(468). Rotator cuff tenocytes have been shown to respond both directly to scaffolds (316) and to other inflammatory stimuli, such as 15-epi LXA₄, an aspirin metabolite (126).

Our results demonstrate that macrophage-secreted cytokines can influence the expression of genes relating to rotator cuff tendon homeostasis. After 48 hours, tenocytes exposed to the 24 hour supernatant from macrophages on Conexa, expressed lower levels of collagen type VI and higher levels of MMP-1 and MMP-3. This observation fits with the increased expression of IL-1 β , TNF- α , IL-6, and IL-10 seen in macrophages on Conexa. IL-1 β is known to up-regulate expression of MMP-1 and MMP-3 in human fibroblasts (469, 470) and has a negative effect on collagen synthesis (471). TNF- α has an inhibitory effect on collagen synthesis in human fibroblasts (472) and IL-6 upregulates expression of MMP-1 and MMP-3 in normal skin fibroblasts (473). Although IL-10 is considered anti-inflammatory, it also decreases collagen expression and increases MMP-3 expression (474).

While cytokines secreted by macrophages on Conexa appeared to promote a catabolic response from tenocytes, this response is not necessarily undesirable. Even though a long-term imbalance in tissue homeostasis may

be detrimental, reorganisation of tissue around the scaffold is likely required for scaffold integration in the short-term. Because we did not observe a difference in gene expression in tenocytes when exposed to the 10 day macrophage supernatant, we suggest that any change in tissue turnover rate is likely short-term. However, the capacity for scaffold-mediated macrophage activation to influence tenocyte behaviour should be acknowledged and explored further.

FBGCs form when macrophages fuse (475), in an attempt to phagocytose a foreign object too big to be engulfed by individual cells (476, 477). FBGC formation is a classic sign of a sustained FBR (399) and they often form on the surface of implanted scaffolds (353). FBGCs secrete degradative enzymes, acidic products, and reactive oxygen species to degrade the scaffold, which can lead to scaffold malfunction (353).

We used an established *in vitro* protocol (425) to encourage FBGC formation by exposure to IL-4 over 10 days. After 10 days, marked differences in FBGC formation between the different scaffolds was observed (figure 4-6 A-F). Specifically, more FBGCs developed on Permacol, compared to GraftJacket and LARS ligament. FBGC formation is material-dependent (478) and influenced by proteins adsorbed (478-480), surface chemistry (481), roughness (482), and topography. Permacol is cross-linked porcine dermis, and cross-linked biological scaffolds have previously been shown to promote FBGC formation *in vitro* (483) and *in vivo* (412, 484, 485)(86). However, FBGCs have also been observed on the surface of non-cross-linked biological

scaffolds (486, 487) and synthetic scaffolds (207, 488) *in vivo* and developed on TCP in the absence of IL-4 stimulation in our study. Similarly to macrophage response, cross-linking may have contributed to the formation of FBGCs in our study but other scaffold properties are also likely to be important.

Our study presents several unique strengths. It is the first study to directly compare the response of human macrophages to allograft, xenograft, and synthetic scaffolds. Utilizing human cells avoids any variation associated with animal-derived primary cells or immortal cell lines. The study also evaluated the effects that macrophage-secreted cytokines have on human tenocytes. Although a previous study has examined this (406), it did not use diseased, patient-derived primary cells in response to four different scaffolds. It is also the first study to demonstrate the *in vitro* formation of FBGCs to commercially available scaffolds.

There are several limitations of our study that must be considered. First, the study was performed *in vitro* and whether our results translate to *in vivo* performance is not clear. Second, we only utilized two cell types even though many more different cell types are involved in inflammatory processes *in vivo*. Third, we only evaluated four scaffolds due to the limited availability of material.

4.7 Conclusions

Our study demonstrates that human macrophages release different amounts of pro-inflammatory cytokines in response to allograft, xenograft, and synthetic scaffolds. We found the greatest expression of pro-inflammatory cytokines in macrophages grown on Conexa, a porcine-derived non-cross-linked scaffold with little residual DNA. These cytokines influenced human rotator cuff tenocytes to increase *MMP* gene expression and reduce *COL6A1* gene expression. While cytokine release decreased over 10 days, scaffolds that induced the highest cytokine levels after 24 hours also showed higher levels at 10 days. This suggests that scaffold signals that promote the inflammatory cytokine expression and release are maintained over time.

Furthermore, we report increased *in vitro* formation of FBGCs on Permacol, a cross-linked porcine dermal scaffold. Even though much work has been done to develop xenograft scaffolds for surgical use, future work should focus on decoupling scaffold characteristics to understand the factors that contribute to changes in macrophage behaviour. In addition, future work should continue to look at the interplay between immune cells, stromal cells, and scaffolds to help create scaffolds that can successfully integrate into the host tissue and promote repair.

Chapter 5 : Discussion

5.1 Thesis aims and hypotheses

The majority of high quality clinical trials of rotator cuff repair surgery reveal that a significant proportion of repairs fail to heal. There has been little progress in the past 20 years in reducing rates of re-tear. A large multicentre randomised trial (the UKUFF trial) reported in 2015 a mean failure rate of 40% with both open and arthroscopic repairs (489). Some surgeons are advocating the use of scaffolds to augment the repair but it is unclear if any of the scaffolds currently in clinical use are effective and reduce the rate of re-tear. Numerous different scaffolds are currently in clinical use. They can be categorised into three main types: synthetic (polymer-derived), allograft (derived from human ECM), or xenograft (derived from animal ECM). The overall aim of this thesis was to evaluate and compare the characteristics of the three different types of existing surgical scaffolds. The data generated is useful not only for informing current clinical practice, but also in the development of future scaffolds.

The first aim of this thesis was to evaluate the mechanical properties of different scaffolds on the macroscale and microscale, and to compare these to the mechanical properties of native supraspinatus tendon. It was hypothesised that the macroscale mechanical properties of synthetic scaffolds would be equivalent to those of the supraspinatus tendon. While the macroscale mechanical properties of the synthetic scaffolds were greater than

those of the allograft and xenograft scaffolds, the properties were inferior to those of the supraspinatus tendon. This was an unexpected finding because Poly-Tape and LARS ligament were originally designed to possess properties similar to the human ACL (490, 491). Strips of ACL 10mm in width failed at forces of $1784 \pm 580\text{N}$ (492), which is stronger than 7mm wide strips of supraspinatus, which failed at an average force of 652N (36). This may have been caused by cutting of the scaffolds into small strips, which could have compromised their mechanical integrity. Also, the scaffolds were thinner and more porous than the supraspinatus tendon which could explain their inferior mechanical properties.

We also hypothesized that the microscale mechanical properties of the allograft and xenograft scaffolds would be equivalent to those of the supraspinatus tendon. The microscale mechanical properties of the allograft scaffold and cross-linked xenograft scaffold were found to be equivalent to the supraspinatus tendon. However, the non-cross-linked xenograft scaffold had a microscale surface stiffness that was greater than the supraspinatus tendon.

The second aim of this thesis was to assess how patient-derived rotator cuff tenocytes behaved in response to different scaffolds *in vitro*. Based upon promising reports in the literature (248, 250, 342), we hypothesized that the human allograft scaffold would stimulate the greatest tenocyte attachment, proliferation, and upregulation of tendon-relevant genes compared to synthetic scaffolds. While allograft scaffolds promoted the greatest

proliferation of tenocytes, synthetic scaffolds, with dense and aligned fibres, supported the highest initial cell attachment. The non-cross-linked xenograft scaffold fostered the largest upregulation of *COL1A1* and *COL3A1* genes. In terms of overall tenocyte behaviour, the biological scaffolds appeared to promote a more favourable response than synthetic scaffolds.

The third aim of this thesis was to better understand how human-derived macrophages respond to different scaffolds *in vitro* and to determine how this response affects the behaviour of rotator cuff tenocytes. Based upon results from previous studies (207, 260), it was hypothesized that the synthetic scaffold would promote the greatest release of pro-inflammatory cytokines, and that this would significantly reduce the proliferation and gene expression of rotator cuff tenocytes. In fact, the non-cross-linked xenograft scaffold prompted the largest release of pro-inflammatory cytokines, which stimulated a reduction in collagen and MMP gene expression in tenocytes.

It was also hypothesized that the synthetic scaffold would foster the formation of the most foreign body giant cells (FBGCs). However, we found that the cross-linked porcine xenograft encouraged the formation of the most FBGCs. These results were slightly unexpected because even with cross-links, ECM-derived scaffolds still present familiar ligands for cell attachment (342). In addition, several studies have reported chronic inflammation associated with synthetic scaffolds (207, 260, 493). Having said this, previous studies have reported increased inflammation in response to cross-linked ECM scaffolds compared to non-cross-linked equivalents (412, 486). However, no

previous study has directly compared the macrophage response between synthetic scaffolds, non-cross-linked ECM scaffolds, and cross-linked ECM scaffolds.

5.2 Properties of scaffolds

5.2.1 Synthetic scaffolds

The synthetic scaffolds assessed in this study, that had dense and aligned bundles of fibres, provided greater tensile strength and suture retention properties compared to the human and porcine dermal scaffolds. These results contrast with previous studies (194, 199), which reported that dermal scaffolds had superior mechanical properties compared to synthetic scaffolds. However, these earlier studies used a weak synthetic scaffold (SportMesh, also known as Artelon) (194, 199) as a comparator, which is not representative of all synthetic scaffolds (12, 314, 315). This raises two important points. Firstly, previous studies have only reported the mechanical properties of scaffolds relative to other scaffolds. If a weak scaffold is compared to a weaker scaffold, it will appear to be strong. This illustrates the value of using a 'gold standard' comparator, which would ideally reflect the properties of the healthy tissue that the scaffold seeks to recapitulate, like the supraspinatus tendon tissue used in this thesis.

Secondly, results from the mechanical chapter demonstrate the important observation that not all scaffolds of the same type have the same properties. For example, even though BioFiber is a synthetic scaffold, its mechanical properties were more similar to the biological scaffolds than the other synthetic scaffolds. This illustrates the need to test specific scaffolds and not draw general conclusions based on the general material from which it is constructed.

We also found that synthetic scaffolds, with dense and aligned bundles of fibres, encouraged the highest initial attachment of rotator cuff tenocytes. These results contrast with previous work that found tenocyte attachment to be highest on human dermal and porcine SIS scaffolds (251, 252). Such inconsistency may be explained by differences in the cells used, with previous studies using cells derived from healthy tissue (251, 252). In contrast, our studies used cells derived from diseased tissue. Differences may also be explained by the variation in the scaffolds used. Previous studies (251, 252) used scaffolds that were different from the scaffolds that we evaluated.

Human-derived macrophages did not mount an overt acute or chronic inflammatory response against the LARS ligament, the PET-derived synthetic scaffold we tested. These results do not align with the conclusions of a study by Grotenhuis *et al.* (260), which found that a PET synthetic scaffold stimulated the largest release of pro-inflammatory response of the four scaffolds tested. This could be explained by differences in the proteins analysed. While Grotenhuis *et al.* (260) measured protein expression of TNF- α , IL-1 β , IL-6, and IL-1RA like we did, they also evaluated several proteins that we did not including MCP-3, RANTES, CCL18, MDC, and MIP-1 α . Specifically, Grotenhuis *et al.* (260) reports a large increase in MIP-1 α (a pro-inflammatory cytokine) in macrophages on the PET synthetic scaffold. MIP-1 α has been associated with increased expression of TNF- α and IL-1 β (494) and therefore may be dysregulated along a similar pathway. However, because we did not measure expression of MIP-1 α , we are unable to determine whether we would have observed similar results. Differences in results

between our study and the study by Grotenhuis *et al.* (260) can also be explained by differences in scaffolds used. Even though Grotenhuis *et al.* (260) also used a PET-derived scaffold, it may have a different molecular weight and may have been processed differently to the PET-derived scaffold (LARS ligament) that we evaluated.

5.2.2 Allograft scaffolds

The human dermal scaffold exhibited poor tensile strength and suture retention, despite previous studies suggesting its superior mechanical properties (194, 199). However, it did exhibit microscale mechanical properties that were equivalent to native supraspinatus tendon and did foster the greatest proliferation of human rotator cuff tenocytes. The ability of a human dermal scaffold to encourage tenocyte proliferation has been reported previously (250, 252) and may help explain its ability to promote healing in animal models (253, 254) and humans (279-281).

Despite having the most native DNA, the human dermal scaffold did not promote a large pro-inflammatory response in human-derived macrophages compared to the xenograft scaffolds tested. Orenstein *et al.* (261) evaluated the response of human macrophages to three different human dermal scaffolds *in vitro* and found significant differences in IL-1B protein expression between them. However, no previous study has directly compared the macrophage response towards human dermal, porcine dermal, and synthetic scaffolds. In a canine rotator cuff model, Adams *et al.* (254) used a human dermal scaffold to augment the repair and found chronic

inflammation after 6 weeks. However, one must re-iterate the problems of using an animal model to evaluate the inflammatory response towards a human-derived scaffold; the animal model will see the human allograft as a xenograft scaffold. Many clinical studies have used human dermal scaffolds for rotator cuff repair (279-284) without any complications or reports of overt inflammation. Furthermore, a recent systematic review by Ferguson *et al.* (495) found that allograft scaffolds were more successful than xenograft scaffolds at promoting healing in human rotator cuff repairs.

5.2.3 Xenograft scaffolds

The two porcine xenograft scaffolds we tested had poor macroscale mechanical properties. This is perhaps unsurprising, given that although the dermis has many functions, bearing high tensile loads is naturally not one of them. Even though one of the scaffolds was cross-linked and the other was not, we reported no differences in the macroscale mechanical properties between them. This conflicts with a previous study by Annor *et al.* (197), which showed that cross-linked dermal scaffolds had greater mechanical properties than non-cross-linked dermal scaffolds. This discrepancy could be caused by differences in number and type of scaffolds used. The non-cross-linked porcine dermal scaffold was stiffer on the microscale than the cross-linked porcine dermal scaffold. This may represent differences in cross-linking or differences in other processing techniques used.

The non-cross-linked porcine dermal scaffold also stimulated the highest expression of *COL1A1* and *COL3A1* genes in rotator cuff tenocytes.

An *in vitro* study by Shea *et al.* (252) found similar results, but also reported increased expression of *DCN* and *SCX-B* genes, which we did not observe. In principle, it is desirable for a rotator cuff scaffold to promote the expression of native tendon ECM genes such as *COL1A1*, *COL3A1*, *SCX-B*, and *DCN*. However, it is unclear whether such *in vitro* observations translate to tangible *in vivo* benefits and a more robust repair. Future work could look to quantify and evaluate the distribution of such proteins following scaffold implantation *in vivo*.

We found significant differences in the response of human-derived macrophages towards the porcine dermal scaffolds compared to human dermal and synthetic scaffolds. The non-cross-linked porcine scaffold stimulated the greatest release of pro-inflammatory cytokines after 24 hours, which caused tenocytes to increase MMP mRNA expression and reduce collagen mRNA expression. These results are at odds with previous work by Orenstein *et al.* (262), which found that cross-linked porcine dermal scaffolds actually promoted a greater pro-inflammatory response than non-cross-linked porcine dermal scaffolds. Differences in results may have been caused by variation between the human monocyte donors, differences in isolation techniques, or differences between the scaffolds tested.

No previous *in vivo* study has reported acute inflammation relating to non-cross-linked porcine dermal scaffolds. This could be because animal and human clinical studies often have later time points (weeks to months) and rarely have an experimental time point within days following scaffold

implantation. While later time points provide an opportunity to evaluate how the scaffold behaves over time, we suggest that an acute time point may help to better understand host-scaffold interactions and better explain observations at later time points. Earlier time points would enable investigators to understand an important window during which inflammation can resolve or be sustained. If inflammation associated with scaffold implantation does not resolve it can develop into chronic inflammation, which is associated with poorer clinical outcomes (271).

Cross-linked porcine dermal scaffold stimulated the formation of the most FBGCs after 10 days. A cross-linked porcine scaffold has been associated with FBGC formation in a previous sheep study (256). Whether the difference in inflammatory response, between the human and porcine dermal scaffolds that we report, translates to clinical performance remains unclear. Many of the clinical studies reporting deleterious inflammatory responses were towards porcine small intestine submucosa SIS (267-269, 271), and clinical studies using porcine dermis (274-277) have been largely positive. Pre-clinical research studies, such as those presented in this thesis, are necessary to investigate this further. Such pre-clinical studies should then be used to inform the design and creation of clinical studies. Such *in vivo* human studies that directly compare human and porcine dermal scaffolds will be of great value to the field.

5.2.4 Biosynthetic scaffolds

Biosynthetic scaffolds appear promising, yet few studies have been published relating to their use or performance. This is the first piece of work to study a commercially available biosynthetic scaffold *in vitro* in the context of rotator cuff repair and to compare it to several other types of commercially available scaffold. It appears that the addition of just one protein (collagen in the case of BioFiber-CM) to a synthetic scaffold is not sufficient to significantly alter the behaviour of tenocytes. It appears that in order to influence cell behaviour, proteins must be organized and presented in a way that they are naturally in the ECM. At present, biosynthetic scaffolds may benefit most from using pieces of decellularised ECM instead of randomly coating the synthetic component in a single protein. While biosynthetic scaffolds show promise, more work needs to be done to understand the most desirable aspects of biological and synthetic scaffolds that can be combined together to form an optimal biosynthetic scaffold.

While several clinical studies have reported favourable outcomes after using synthetic scaffolds for rotator cuff repair (286-288, 290-293) few studies have compared them to other scaffolds that are available on the market. For the first time, this thesis has compared the performance of several commercially available synthetic scaffolds to the performance of several biological scaffolds. The work highlights variation within and between different scaffold types.

5.3 Limitations of current scaffolds available for rotator cuff repair

None of the scaffolds examined in this thesis were specifically designed to augment a surgical rotator cuff repair. Poly-Tape and LARS ligament were originally designed as ACL ligament replacement devices (490, 491). The first clinical studies using GraftJacket were for diabetic wounds (496) and Achilles repair (497). One of the first uses for Permacol was in bladder surgery (498). BioFiber (499), BioFiber-CM (499), Conexa (33), and X-Repair (500) are indicated for the surgical repair of several tissues including rotator cuff, Achilles, quadriceps, and patellar.

Table 5-1 lists published clinical studies that have used these scaffolds to repair different anatomical sites. While a scaffold may not elicit a negative response when used for a variety of clinical needs, it is unlikely to be optimal to enhance or enable repair of a range of tissues with fundamentally different mechanical and biological requirements. This may help to explain why so many commercial scaffolds have so little clinical evidence to support their use.

Table 5-1. Reported clinical uses of commercially available scaffolds used in this study.

Scaffold name	Reported site of clinical use
X-Repair	Rotator cuff (288, 289)
LARS ligament	Anterior cruciate ligament (490, 501) Posterior cruciate ligament (502, 503) Collateral ligament (504) Knee extensor mechanism (505) Rotator cuff (506)
Poly-Tape (formerly Leeds-Keio)	Rotator cuff (507) Medial patellofemoral ligament (508, 509) Achilles (510, 511) Knee extensor mechanism (512) Iliofemoral ligament (513) Lateral ankle ligament (514) Quadriceps tendon (515) Patellar tendon (516)
BioFiber	No clinical studies reported
BioFiber-CM	No clinical studies reported
GraftJacket	Wound coverage (496, 517, 518) Abdominal wall (519, 520) Achilles (497, 521-524) Rotator cuff (295, 525-528) Peroneal tendon (529)
Permacol	Abdominal wall (530, 531) Vagina (532) Hernia (533) Parotid gland (534) Rotator cuff (278, 294) Trapeziectomy (535)
Conexa	Achilles (536) Rotator cuff (274, 275)

5.4 Implications of this work on scaffold design

5.4.1 Ideal properties of scaffolds for rotator cuff repair

Many scaffolds currently being used for rotator cuff repair were not originally designed for this purpose. The results from our studies demonstrate that existing scaffolds do not have many of the characteristics that would help augment surgical rotator cuff repairs. To design more effective rotator cuff scaffolds in the future, it is helpful to formally define the properties that are most desirable.

Table 5-2. Ideal properties of a scaffold for rotator cuff repair.

Type of property	Property	Reasoning	Method of evaluation
Mechanical	High tensile strength	Provide mechanical support, particularly for massive tears	Tensile mechanical testing
Mechanical	High suture retention	Minimize most common form of repair failure	Tensile mechanical testing
Mechanical	Appropriate macroscale stiffness	Provide some flexibility while resisting gap formation and cyclic elongation	Tensile mechanical testing
Mechanical/biological	Appropriate microscale stiffness	Provide appropriate biophysical cues to host cells	Atomic force microscopy
Structural	Flexibility	Can be trimmed to size for specific patient's need and for arthroscopic repair	Can fit within arthroscopic portals
Structural	Thin	Prevent impingement on coracoacromial arch	Micrometer measurements
Structural	Porous	Enable host cell infiltration and incorporation of scaffold into native tissue	Micro-CT analysis
Structural	Absorbable	Gradually replaced by host tissue to avoid chronic inflammatory response	Animal model or bioreactor
Biological	Bio-instructive	Promotes native tenocytes to create organized tissue, similar to original tendon ECM	<i>In vitro</i> tissue culture, animal model, or clinical study
Biological	Immunomodulatory	Modulate immune cells to avoid overt inflammatory response and host rejection	<i>In vitro</i> tissue culture, animal model, or clinical study

5.4.2 Mechanical properties

It is important to design a scaffold with the macroscale mechanical properties to cope with the forces experienced following rotator cuff surgery. Polymers are an attractive base material because they can be manipulated easily to create a scaffold with the specific mechanical properties. In terms of scaffold structure, the work from this thesis suggests that tightly woven and aligned bundles of synthetic fibres provide good strength, suture retention, and promote initial cell attachment. This porous design also permits tenocyte invasion, which may lead to a more rapid tissue integration.

Synthetic scaffolds can be woven into a variety of different configurations. Similar to clothing fabrics, different weaving patterns can alter the properties of the scaffold such as strength and porosity. Even though numerous weaving patterns exist, few studies have extensively explored the different options available that could be used for a rotator cuff scaffold. Different weaving patterns could be used to create scaffolds with specific macroscale mechanical properties to meet the demands of a specific tissue. Furthermore, weaving provides an opportunity to make a multi-phased scaffold, which combines different types of weaves into one scaffold. This would be advantageous when trying to re-create the multi-phased rotator cuff enthesis, which is a complex tissue with distinct tissue zones. Specific zones could be coupled with different ECM components or growth factors to promote the development of the tissue normally associated with that zone. For example, the tendon portion of the enthesis could be made from aligned synthetic fibres and impregnated with TGF- β to encourage tenocyte alignment and

collagen expression. This could be connected to an inter-woven component that would represent the criss-crossing collagen fibres in the uncalcified fibrocartilage portion of the enthesis. This portion could also be seeded with bFGF (537) to encourage chondrocytes to increase GAG content. The next enthesis portion is the calcified fibrocartilage, which would be similar to the uncalcified fibrocartilage, perhaps with the addition of BMP-2 to encourage partial calcification (538).

Absorbable polymers, such as poly-lactic acid, are attractive because they are strong, but gradually degrade into metabolites that are naturally secreted by the body. This enables host tissue to gradually replace the scaffold and reduce the likelihood of complications associated with chronic inflammation (539). It is likely that the mechanical requirements of a scaffold will diminish over time as native cells and ECM populates the material, but we do not know what the ideal rate of scaffold degradation should be. This will likely vary depending upon the age and health of the patient, in addition to the type of rehabilitation protocol used.

Even though suture pull-out is the most common mode of rotator cuff surgical failure (200), little work has been done to create scaffolds that address this issue. Almost every surgical repair technique attaches the scaffold by suturing around its periphery. Future scaffolds could be designed to have a thicker outside edge, or specific points for suture attachment (like eyelets), to reduce the rate of suture pull-out. Sutures could also be created to be flatter

and ribbon-like, to encourage downward compression of the repair and reduce stress concentration at the suture-scaffold interface.

5.4.3 Tenocyte response

As the most common cell type in tendon and the main producer of ECM proteins, rotator cuff scaffolds must be designed to promote a favourable response from tenocytes. Our data suggests that non-cross-linked ECM-derived scaffolds are most capable of influencing tenocyte behaviour. Even though many exciting techniques, such as electrospinning and 3D printing, are available to manipulate synthetic materials, no current technique can recreate the complexity of the native ECM. Furthermore, the similarity in tenocyte behaviour between BioFiber and BioFiber-CM (BioFiber coated with bovine collagen) in chapter 3 demonstrates that altering the tenocyte response cannot be achieved by simply adding one protein.

It is more likely that ECM ligands are arranged in a way that influence cell behaviour by binding cells in the manner that they would bind *in vivo*. Incorporating an ECM component into future scaffolds may offer the best chance of promoting the proliferation and collagen expression of a diseased and inactive tenocyte population. However, the tissue from which the ECM component should be derived and how it should be decellularised is not clear. Various processing techniques are used during the decellularisation process of ECM tissue during the creation of biological scaffolds. However, little is known about how these different processing techniques influence the

composition and structure of the resulting ECM, and subsequently how this affects tenocyte behaviour.

Studies that systematically evaluate different processing techniques individually and in combination would be of great value. This would help to determine an optimal series of processing techniques required to generate an ECM that stimulates favourable cell behaviour. Although many tendon researchers put value in stimuli that upregulate tendon ECM proteins, little work has been done to understand how scaffolds influence the way that these ECM proteins are being arranged in the surrounding environment. It is likely that the positioning, not just the quantity, of ECM components is important to successful rotator cuff tendon healing. Scaffolds may have the capacity to influence this process, yet little work has been done to designs scaffolds to optimize their influence.

While a thin layer of rotator cuff tendon ECM is a logical choice, it is possible that ECM from a different tissue is more effective at promoting a healing response. To make that decision, a greater understanding of the underlying mechanisms that govern tenocyte-ECM interactions is required. Such work could help identify desirable components or design features of the ECM that could be selectively maintained, while unnecessary proteins could be discarded. Alternatively the desirable components could be removed and selectively attached to the synthetic component of the scaffold.

The favourable response of cells towards ECM scaffolds could be due to biochemical cues from bound growth factors, not just biophysical ones.

GraftJacket is thought to contain high concentrations of native fibroblast growth factor (FGF) and we observed increased cell proliferation on GraftJacket. Previous studies have used scaffolds to deliver growth factors (540-542). However, growth factors are rapidly used or degraded, and the required growth factors vary in space and time. For example, macrophages initially secrete TGF- β (56) during the first 7 days after a rotator cuff tear, but this is reduced as the inflammation is resolved over time. One could imagine a multi-phased scaffold that contains growth factors specific to each phase of the enthesis. Also, the release of these growth factors can be controlled temporally, such that growth factors are released only during the stage of repair in which they are needed. Microspheres can be used to deliver growth factors and the release kinetics can be controlled by altering the thickness and material type (541, 543). This is especially important when trying to promote a healing response in elderly patients with massive chronic tears. This is because their native tendon cell population is less proliferative and contains fewer stem cells than younger patients (544). This means that that signals must be potent to have any chance of these cells mounting a repair response.

For some patients, especially the elderly, it is unlikely that existing treatment techniques including scaffold-augmented repair could ever promote a biological repair. For these patients, a tendon replacement may be an attractive therapeutic option. Scaffolds have been created to replace anterior cruciate ligaments, but the replacement of a tendon is more complex because of its role in transferring forces from muscle to bone. The creation of a scaffold that replaces the native tendon is an attractive option that warrants

further investigation. Such a device would need to be integrated into the host tissue, so work presented in this thesis relating to tenocyte and macrophage response provides a platform for this future work.

5.4.4 Inflammatory response

Several studies have highlighted the presence of inflammatory cells and the importance of inflammation in the healing of rotator cuff tendons (126, 267, 271). Our work demonstrates that scaffolds possess the capacity to not only influence the behaviour of macrophages, but also that this subsequently affects the behaviour of surrounding tenocytes. Thus, designing a scaffold that modulates the response of inflammatory cells would be desirable.

The results from chapter 4 suggest that xenografts promote a greater pro-inflammatory response in human-derived macrophages compared to allografts and synthetic scaffolds. Thus, current evidence suggests that if ECM is to be used, it should be of human origin. While our results indicate a species-specific response, we cannot rule-out the possibility that different processing techniques influenced these results. The precise processing techniques used by the manufacturers of scaffolds for human use are invariably not disclosed and are kept secret as essential know how by the company. A future investigation could take human and porcine dermal tissue, process them in the same manner, then evaluate the inflammatory response using our *in vitro* macrophage model. By controlling for processing techniques, this would help determine whether there is a species-specific inflammatory response towards biological scaffolds.

A greater understanding of which ECM components influence inflammatory cells and how they influence them would be of great value to the field. As discussed above, all biological scaffolds are processed in some way to allow decellularisation, but we do not understand how these different processing techniques impact ECM components and how these changes subsequently influence inflammatory cells. Further interrogation of this would help to develop processing techniques that kept desired ECM components while removing inflammation-causing antigens. Furthermore, in relation to all scaffold types, there is an increasing body of work demonstrating that alterations in the microscale mechanical and structural properties of materials can influence the behaviour of inflammatory cells (420)(47). However, this has not been fully explored in the context of scaffolds for rotator cuff tears and warrants further investigations.

Scaffolds should be designed in light of the unique inflammatory environment in the shoulder. Following implantation, scaffolds are subject to a foreign body reaction (FBR), mounted by the patient's immune system. This is a sequential process made up of a series of specific steps. It is likely that some inflammation is necessary and beneficial to rotator cuff tendon healing. However, how much and for how long is not clear. Scaffolds have the potential to spatially and temporally regulate inflammation and the FBR within the shoulder, but few studies have addressed this important issue. Designing scaffolds with biochemical and biophysical cues that regulate biological mechanisms in space and time could help to recreate complex tissues such as the enthesis in the rotator cuff tendons.

5.5 Future work

Future scaffolds should be designed with a specific tissue and purpose in mind. To augment the surgical repair of large-massive rotator cuff tears, future scaffolds should account for the biological and mechanical demands of the healing tissue. The fact that no commercially available scaffold does this may help explain their limited clinical efficacy.

5.5.1 Scaffold attachment techniques

The success of rotator cuff scaffolds in the future may depend upon attachment technique, not just scaffold design. Previous cadaveric studies have demonstrated differences in the mechanical properties of rotator cuff scaffold attachment techniques (216, 217). However, no study has explored this using an *in vivo* model. Specific attachment techniques could increase the mechanical properties of the repair, but could also influence how the scaffold interacts with the native tissue. If scaffolds are designed to influence biological processes in the host tissue, scaffolds should be attached in a manner that maximises this interaction. Further *in vivo* work focusing on scaffold attachment techniques could be of great value.

5.5.2 Scaffold pre-conditioning

The fact that the patient is having a surgical rotator cuff repair indicates that their native repair mechanisms alone were not able to restore function during conservative treatment. Scaffold augmentation provides an opportunity to deliver biological agents to enhance these native repair mechanisms. Scaffolds could be pre-seeded with cells and/or growth factors prior to implantation to

direct changes in certain biological processes. Previous work has demonstrated that a provisional matrix of serum proteins spontaneously forms on the surface of a scaffolds following implantation; the characteristics of which are largely determined by the chemistry of the scaffold (353). Pre-coating the scaffold with specific proteins/growth factors would allow for greater control over this process. However, the type and amount of signal required will likely change over the course of the repair process. To overcome this issue, scaffolds could be designed to release certain growth factors at specific time points. Alternatively, scaffolds that persist over time could be used as targets for the injection of biological factors using ultrasound guided injections.

Despite the theoretical therapeutic benefit, little is known about which biological agents to deliver, how much to deliver, when to deliver them, and how best to deliver them. Future work should focus upon understanding these issues better to improve the healing rate of scaffold-augmented repairs.

5.5.3 ECM processing

ECM-based scaffolds are popular for augmenting the surgical repair of many tissues, including the rotator cuff. During manufacturing, such scaffolds experience a range of different processing techniques. However, little is known about the effects that different processing techniques have upon the scaffold final structure and how this subsequently influences cell behaviour. This information is not readily available because all commercial scaffolds are produced using a set of proprietary techniques, so cause and effect responses

of different processing techniques cannot easily be determined. Systematic studies that compare different processing techniques and combinations of processing techniques in a standardized way are required. Such studies would help to determine the optimal combination of processing techniques that can minimize foreign antigens, while maintaining the proteins and structures that promote a healing response.

5.5.4 Pre-clinical testing

Even if scaffolds are designed to address specific needs of a target tissue, rigorous pre-clinical testing is required to evaluate whether these goals have been achieved.

5.5.4.1 Bioreactors

In tissue engineering, a bioreactor is an *in vitro* device that uses mechanical processes to more accurately mimic the natural *in vivo* environment (545). A bioreactor could be used to more accurately model human rotator cuff tears. This would help in the development and testing of more effective scaffolds. The development of bioreactors is particularly important given that existing small animal models fail to faithfully model human rotator cuff tears. While most human rotator cuff tears develop over time and are most common in elderly patients, animal models surgically sever a healthy tendon in a young animal with much greater healing potential. Furthermore, most animal models are quadrupeds that weight-bear during locomotion, which is not the case in bi-pedal humans. In addition, most animal shoulders have tendons that insert independently onto the humeral head, whereas human tendons inter-digitate to form a continuous cuff at the insertion. Such fundamental

differences may hinder the development of effective scaffolds to treat rotator cuff tears.

In the absence of good small animal models, rigorous *in vitro* techniques become more valuable. One of the major strengths of standard *in vitro* tissue culture testing is that patient-derived primary cells can be used to more accurately model the response seen *in vivo*. One limitation is the absence of dynamic loading that would normally occur *in vivo*. Bioreactors offer a valuable opportunity to mechanically load cells *in vitro* in a manner that they would experience naturally. Creating a bioreactor that accurately replicates the anatomy and forces experienced by a human rotator cuff would help to develop new rotator cuff scaffolds.

5.5.4.2 Large animal models

Despite their utility, bioreactors are not able to accurately replicate complex *in vivo* processes such as the chronic inflammatory response. Large animal models provide an opportunity to study the inflammatory reaction towards implanted scaffolds over time. Such an animal model would be particularly helpful in understanding how scaffolds influence the chronic inflammatory response of native tendon cells—something which has not been done previously, but warrants further investigation. Ideally, such large animal models would be non-human primates because they are the most similar to humans in terms of genetics, cell response, and biomechanical loading. However, such animal models are expensive and may present ethical concerns.

5.5.5 Clinical studies

Following rigorous pre-clinical studies, well-designed clinical studies should be conducted before releasing the scaffold onto the market. To this end, we have started a small observational clinical study called SECURE:

5.5.5.1 Scaffold Evaluation for Cuff REpair (SECURE) study

The SECURE study is an on-going clinical study being conducted to evaluate the tissue response and efficacy of commercially available rotator cuff scaffolds. The study is a small observational study in humans, where suitable rotator cuff tear patients are selected to undergo surgical rotator cuff repair with or without scaffold augmentation. In the scaffold-augmented group, they can receive an allograft, xenograft, or synthetic scaffold. A tissue biopsy is taken at the time of surgery and an ultra-sound guided needle biopsy is taken 4 weeks following surgery. The biopsy tissue will be evaluated histologically for tenocyte and inflammatory cell response, and these results will be correlated with standard clinical evaluations (Oxford Shoulder Score) and mechanical force testing. The SECURE study provides a fantastic opportunity to determine how well our *in vitro* investigations mimic the response seen *in vivo*.

5.5.5.2 Randomized controlled trials

While the SECURE study is a valuable step towards developing better rotator cuff scaffolds, large randomized-controlled clinical trials are required. Many commercially available scaffolds lack robust clinical data and trials comparing several scaffolds would help to determine which scaffolds are most effective.

Such studies would also help identify favourable features of existing scaffolds that could be incorporated into the design of future scaffolds.

5.6 Limitations of this work

There are several limitations to this thesis that must be considered. The first limitation is that we are unable to definitively explain the mechanism underlying many of our observations. This is because there are many aspects of scaffolds that could influence mechanical properties and cell response, but we were not able to de-couple these. Therefore, we could not determine what effect, if any, certain scaffold parameters had upon our observed results.

The second limitation of this work is that it is unclear how accurately the *in vitro* results will reflect *in vivo* performance. While the general conclusions of this work aligns with many previous studies, we cannot say that the behaviour we observed *in vitro* will be seen *in vivo*.

The third limitation is that we were unable to test all eight scaffolds across all 3 chapters. Unfortunately, we had a limited supply of scaffolds and many suppliers were unwilling to provide us with additional scaffolds when our results did not align with what they were hoping for. This thesis would have been a more comprehensive evaluation if we were able to achieve this.

The fourth limitation is that the cell work was performed in static tissue culture. The shoulder is an active and mobile joint that contains cells that respond to mechanical loading. Future work could benefit from mimicking the forces naturally experienced during the recovery from a surgical rotator cuff repair.

5.7 Key findings of this thesis

Surgical repairs of rotator cuff tears have a high rate of re-tearing. In an effort to reduce re-tear rates, some surgeons use scaffolds to augment the repair. Even though many scaffolds are commercially available, few have demonstrated convincing clinical efficacy. The aim of this thesis was to evaluate and compare the mechanical properties, tenocyte response, and macrophage response of several commercially available scaffolds. From these studies, we looked to determine desirable attributes of existing scaffolds that could be used to develop better scaffolds in the future. In the process, we also wanted to develop a series of *in vitro* tests that could be used to rigorously assess and compare current and future scaffolds.

Even though synthetic scaffolds had superior macroscale mechanical properties compared to xenograft and allograft scaffolds, those properties were inferior compared to the supraspinatus tendon. While the allograft scaffold had poor macroscale mechanical properties, it promoted the greatest proliferation of rotator cuff tenocytes. The non-cross-linked xenograft scaffold also had weak macroscale mechanical properties, but promoted the greatest increase in expression of collagen genes in rotator cuff tenocytes.

The non-cross-linked xenograft and cross-linked xenograft promoted the greatest acute and chronic inflammatory responses, respectively. In contrast, the macrophage responses against the allograft and synthetic scaffolds were relatively minimal.

This work highlights significant variation in the properties and performance of different commercially available scaffolds. It also reveals that none of the existing scaffolds tested meet all of the requirements to successfully augment a surgical rotator cuff repair. This may explain why there is so little clinical evidence to support the use of scaffolds that are currently available. We suggest that future scaffolds be specifically designed to meet the mechanical and biological demands of the target tissue. The pipeline of *in vitro* tests developed in this thesis could be used to screen potential scaffolds and help determine which scaffolds to evaluate in large animal models and human trials.

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Appendices

Appendix A: Precise rheometry measurements of storage modulus, loss modulus, and calculations for Loss tangent. Each row represents a separate value.

Storage modulus (Pa)	X-Repair	LARS Ligament	Poly-Tape	BioFiber	GraftJacket	Permacol	Conexa	Supraspinatus
1	456623.67	470095.33	451866.33	224267.33	26674.77	149436.67	62548.27	129057.67
2	576725.00	561090.33	413422.33	228819.67	26209.07	155611.00	56941.93	183611.33
3	433073.33	477198.33	394137.33	276575.33	28169.93	144159.67	52547.43	72645.63
4	462155.67	418053.00	396062.33	222229.00	24877.73	157867.00	61648.80	357588.00
5	723212.67	489807.00	524934.00	185563.67	28970.67	183259.67	51763.03	135948.00
Loss modulus (Pa)	X-Repair	LARS Ligament	Poly-Tape	BioFiber	GraftJacket	Permacol	Conexa	Supraspinatus
1	47928.80	67055.13	113458.00	14206.40	6944.44	28064.10	9622.36	44656.77
2	60329.33	80849.10	129452.67	16359.30	6455.62	29265.67	8469.05	56859.30
3	38521.73	73538.83	107297.70	15707.97	6748.67	29580.97	9297.65	24217.07
4	46408.50	65265.20	107759.33	15302.10	5973.36	30751.90	10997.29	152206.67
5	55879.10	83764.33	150379.67	14150.17	6087.01	32892.60	8428.65	44466.47
Loss tangent	X-Repair	LARS Ligament	Poly-Tape	BioFiber	GraftJacket	Permacol	Conexa	Supraspinatus
1	0.10	0.14	0.25	0.06	0.26	0.19	0.15	0.35
2	0.10	0.14	0.31	0.07	0.25	0.19	0.15	0.31
3	0.09	0.15	0.27	0.06	0.24	0.21	0.18	0.33
4	0.10	0.16	0.27	0.07	0.24	0.19	0.18	0.43
5	0.08	0.17	0.29	0.08	0.21	0.18	0.16	0.33

Appendix B: The sequences and validations of mRNA primers used.

Gene symbol	Acc no.	Forward primer	Reverse primer	Amplicon length	**R ²	efficiency
IL6	NM_000600	AGTAACATGTGTGAAAGCAGCA AAG	GGCAAGTCTCCTCATTGAATC C	234	0.998 9	90.8
TNF alpha	NM_000594	GGCCAAGCCCTGGTATGAG	TAGTCGGGCCGATTGATCTC	92	0.981 5	98.0
IL12b	NM_002187	AGAAGATGGTATCACCTGGACC	GAACCTCGCCTCCTTTGTGAC	127	0.997 2	95.6
IL-8	NM_000584	TGCTAAAGAACTTAGATGTCAGT GCAT	TGGTCCACTCTCAATCACTCTC A	93	0.999 8	93.8
IL10	NM_000572	TACGGCGCTGTCATCGATT	GGCTTTGTAGATGCCTTTCTCT TG	103	0.996 4	101.6
ACTB	NM_001101	CCTGGCACCCAGCACAAT	GCCGATCCACACGGAGTACT	69	0.995 3	93.5

**R² is the coefficient of variance between primer replicates.